

**CERVICAL RIPENING WITH PGE₂ FOR
TRANSCERVICAL EMBRYO TRANSFER IN SHEEP:
STUDIES OF EP₃ RECEPTOR mRNA**

This thesis is presented for the degree of Doctor of Philosophy (PhD)
in the Faculty of Veterinary Medicine of the University of Glasgow

by

Lina Audicana,
DVM (Zaragoza), MVM (Glasgow), MRCVS

Research conducted in the Reproductive Research Group,
Departments of Veterinary Anatomy and Physiology,
University of Glasgow Veterinary School,
Bearsden Road,
GLASGOW G61 1QH.

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ABSTRACT

Artificial insemination (AI) and embryo transfer (ET) in the ewe are severely restricted by the rigid, impassable cervix that resists catheterization. This thesis deals with the ripening effect of intravaginal prostaglandin E₂ (PGE₂) in the luteal phase ovine cervix. The potential of this drug to induce cervical ripening has been extensively studied in periparturient sheep and other mammalian species to facilitate labour, but not as a mean of softening the cervix during the cycle. Physiological cervical ripening is characterised by macroscopical, ultrasonographical, microscopical, mechanical and biochemical changes. Studies during pregnancy and at term suggest that PGE₂ mimics these changes, but there are no studies during the cycle. The sensitivity of the cervix to PGE₂ during the cycle may be lower than at term, but there have been no attempts to characterise the PGE₂ receptors (EP₁-EP₄) in the cervix.

The ultrasonographic appearance of the cervix has been described in other species, but not in sheep. In chapter V, ultrasound is evaluated in the monitoring of cervical changes during the cycle, the ovine cervix was imaged at oestrus and during the early luteal phase. This was achieved employing the transrectal technique and two probes of 5 MHz and 7.5 MHz (Toshiba) and a linear scanner (Capasee, Toshiba).

In chapter III, several sheep in the luteal phase and early pregnancy (n=15), were treated with different regimes of PGE₂ alone or together with oestradiol, to induce cervical ripening. Catheterization was incomplete, but deeper scores (4 cm) than in control sheep (0.1-3 cm) were achieved. Following PGE₂ treatment on day 6 of the cycle, microscopical changes similar to an inflammatory response and therefore similar to those during labour were observed. There was activation of polymorphonuclear leukocytes (PML), eosinophils, mast cells and platelets. Vasodilatation and extravasation of red blood cells were also prominent.

The findings in chapter III, suggested that there was a response to PGE₂ during the luteal phase and that receptors for PGE₂ may be present in the cervix. The observation of PML degranulation is in line with current theories of physiological cervical ripening. These cells, when activated, release collagenolytic enzymes, like elastase, MMP-8 and MMP-9 capable of softening the cervix by degrading the extracellular matrix. It was hypothesised here that the EP₃R, one of the four PGE₂ receptor subtypes, may be involved in the activation of PMLs.

At the beginning of these studies, the only reagents available for the characterisation of the EP₃R in the ovine cervix were a few cDNA sequences in other mammalian species. The first objective therefore was to isolate and sequence cDNA encoding for the ovine EP₃R, using RT-PCR techniques and bovine primers. The successful isolation and sequencing of a cDNA from kidney, using RT-PCR, is described in the first part of chapter IV. The sequence of the fragment isolated was highly homologous to the bovine cDNA sequence, indicating very strongly that it corresponded to the ovine homologue.

In the second part of chapter IV, mRNA expression studies are described. The same primers as the cDNA isolation were used to study mRNA expression, DNase I incubations of the RNA were necessary because the fragment amplified was found to be intronless. Using this method, the presence of mRNA was demonstrated in kidney, liver, uterus, adrenal gland and skin. This distribution pattern is similar to other species and further confirms the identity of the cDNA fragment as the ovine EP₃ receptor. Expression of this message was also found for the first time in the oestrous and luteal cervix, by RT-PCR and on the periparturient cervix by Southern analysis. These preliminary data suggest that the EP₃R may have a function in the ovine cervix. It is reasoned, based on these findings and other publications, that EP₃R may act as a proinflammatory receptor, activating the cellular response observed in chapter III.

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ABBREVIATIONS:

ABP: androgen binding protein
A1PI: a1 proteinase inhibitor (former antitripsin)
AI: artificial insemination
bp: base pairs
cAMP: cyclic adenosin monophosphate
cATP: cyclic adenosin triphosphate
cDNA: complementary DNA
cGMP: cyclic guanosin monophosphate
C5a: the active fragment of the complement
DHAS: dehydroepiandrosterone sulphate
DNA: deoxyribonucleic acid
DNase I: deoxyribonuclease I
dNTP: deoxynucleotide triphosphates (dATP, dCTP, dGTP and dTTP)
ddNTP: dideoxynucleotide triphosphates (ddATP, ddCTP, ddGTP and ddTTP)
DP or DPR: prostaglandin D receptor
DTT: dithiothreitol
DSPG-II: dermatan sulphate proteoglycan-II
eCG: equine chorionic gonadotrophin
E1: oestrone
E2: oestradiol-17 β
ER: oestrogen receptor
ERc: cytosolic ER
ERn: nuclear ER
EP or EPR: prostaglandin E₂ receptors
EP₁ or EP₁R: subtype EP₁ of the prostaglandin E₂ receptors
EP₂ or EP₂R: subtype EP₂ of the prostaglandin E₂ receptors
EP₃ or EP₃R: subtype EP₃ of the prostaglandin E₂ receptors
EP₄ or EP₄R: subtype EP₄ of the prostaglandin E₂ receptors
ET: embryo transfer
fMLP: N-formyl-methionyl-leucyl-phenilalanine
FP: prostaglandin F receptor
GAG: glycosaminoglycan
GPCR: G protein coupled receptors
HETE: hydroxyeicosatetranoic acid

HLE: human leukocyte elastase
 hTP or hTPR: human thromboxane A₂ receptor
 IL-1: interleukin 1
 IL-1 β : interleukin 1 β
 IL-5: interleukin 5
 IL-8: interleukin 8
 IP or IPR: prostaglandin I receptor
 IP3 or Ins P3: inositol triphosphate
 LM: light microscopy
 LTB₄: leukotriene B₄
 LTC₄: leukotriene C₄
 MCP-2: monocyte chemotactic protein-2
 MCP-3: monocyte chemotactic protein-2
 MFA: meclofenamic acid
 MIP-1 α : macrophage inflammatory protein α
 MMP: matrix metalloproteinase
 proMMP: proenzyme of MMP
 MMP-1: interstitial collagenase
 MMP-2: gelatinase A
 MMP-3: stromelysin-1
 MMP-8: PML collagenase
 MMP-9: gelatinase B
 MOET: multiple ovulation and embryo transfer
 OTR: oxytocin receptor
 PAF: platelet activating factor
 PCR: polymerase chain reaction
 PG: prostaglandin
 PGE₂: prostaglandin E₂
 PGF_{2 α} : prostaglandin F_{2 α}
 PGHS: cyclooxygenase prostaglandin H synthase (COX: cyclooxygenase)
 PGHS-1: constitutive form of PGHS (COX-1: constitutive COX)
 PGHS-2: inducible form of PGHS (COX-2: inducible COX)
 PGI₂: prostacyclin
 PKC: protein kinase C
 PML: polymorphonuclear leukocyte
 P4: progesterone
 PR: progesterone receptor
 nPR or PRn: nuclear progesterone receptor

RACE: rapid amplification of cDNA ends
RBC: red blood cell
RLX: relaxin
pRLX: porcine relaxin
RNA: ribonucleic acid
RU 486: Mifepristone (antiprogesterone)
mRNA: messenger RNA
ROI: reactive oxygen intermediates
RT: reverse transcription (cDNA synthesis)
RT-PCR: reverse transcription- polymerase chain reaction
TEM: transmission electron microscopy
TIMP: tissue inhibitor of MMPs
TIMP-1
TIMP-2
TIMP-3
TM: transmembrane domains
TNF: tumour necrosis factor
TNF α :
TxA₂: thromboxane A₂
TP or TPR: thromboxane A₂ receptor
SEM: scanning electron microscopy
SLPI: secretory leukoprotease inhibitor
WBC: white blood cell
ZK 98.299: Onapristone (analogue of RU 486)
ZK 98.734: Lilepristone (analogue of RU 486)

DECLARATION

I declare that this thesis is the sole work of the author except where acknowledged in the text.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 THE ANATOMY OF THE OVINE CERVIX: IMPORTANCE AT OESTRUS AND DURING THE EARLY LUTEAL PHASE FOR ARTIFICIAL INSEMINATION (AI) AND EMBRYO TRANSFER (ET).

The cervix uteri is a thick walled fibromuscular tube, extending from the internal uterine orifice to the external uterine orifice and connecting the body of the uterus with the vagina. The morphology of this organ can vary markedly between species, reflecting differences in copulation, sperm transport, parturition and embryonic development (Hafez, 1973, Kanagawa and Hafez, 1973; Meiter and Fentener van Vlissingen, 1993; Maltier et al., 1993b). The cervix uteri, uterus proper and cranial vagina, evolve from fusion of the right and left paramesonephric or Müllerian ducts in a caudocranial direction and different degrees of fusion can be observed through evolution (Meiter and Fentener van Vlissingen, 1993; Maltier et al., 1993b). This explains the existence of species differences in the type of uterus: duplex (marsupials and rabbits), semiduplex (rats), bicornuated (ungulates and carnivora), bipartite (pigs) and simplex (primates). Similarly, as a result of insufficient fusion of the ducts, congenital abnormalities are commonly observed, as the duplicity of the cervix and vagina in ewes and cows (Hafez, 1973; McEntee, 1990).

The ewe has a single cervix, which in the non-pregnant animal measures 5-10 cm long and 2-3 cm wide (see Plate 1.1). The lumen of the cervical canal is narrow in most species, but in the sheep and other ruminants (cow and goat) it is also very tortuous due to the presence of mucosal folds (Plate 1.2 B) (Hafez, 1973). In the ewe, the toughness, the shape and the orientation of these folds render the cervical canal impassable with rigid instruments during the luteal phase and also during oestrus, despite the slight relaxation that occurs under the influence of oestrogens (El-Banna and Hafez, 1972).

This has practical implications for artificial insemination (AI) and embryo collection and transfer (ET). In cattle, non-surgical techniques have been developed, involving the passage of a stainless steel catheter through the cervical canal. This type of catheterization is difficult in the goat and impossible in the sheep. The lumen is smaller and the folds are more numerous and less concentric, especially in the sheep. Transrectal manipulation of the cervix, which helps to guide the catheter, is not possible either in the small ruminants. Artificial insemination (AI) with fresh semen is intracervical rather than transcervical in the ewe. AI with frozen semen and ET require access to the uterus and therefore laparoscopy is required (Evans and Maxwell, 1987; Nellenschulte and Niemann, 1992). Laparoscopic AI and ET are invasive techniques and have to be carried out by a veterinary surgeon, this adds to their cost and limits the commercial application of the techniques (Stubbings, 1991). Laparoscopic ET has repeatability problems; from the fourth flushing lower recovery rate is observed and improper healing of the uterine wounds may occur (Nellenschulte and Niemann, 1992). There is also concern from the welfare point of view, this makes the search for non-invasive techniques a priority (Stubbings, 1991).

The drawbacks of laparoscopic AI and ET and the unavailability of an alternative transcervical technique, is restricting the breeding potential of sheep by preventing the export of genetic material, breed improvement programmes, disease control and research. As a consequence, the research on the cervix has concentrated on studying the structure of this organ, the aim being to improve AI and ET techniques.

Plate 1.1

Gross anatomy of the ovine reproductive tract at the early luteal phase. 1: external os, 2: mid cervix and 3: internal os of the cervix uteri. 4: corpus uteri, 5: uterine cornua and 6: ovaries

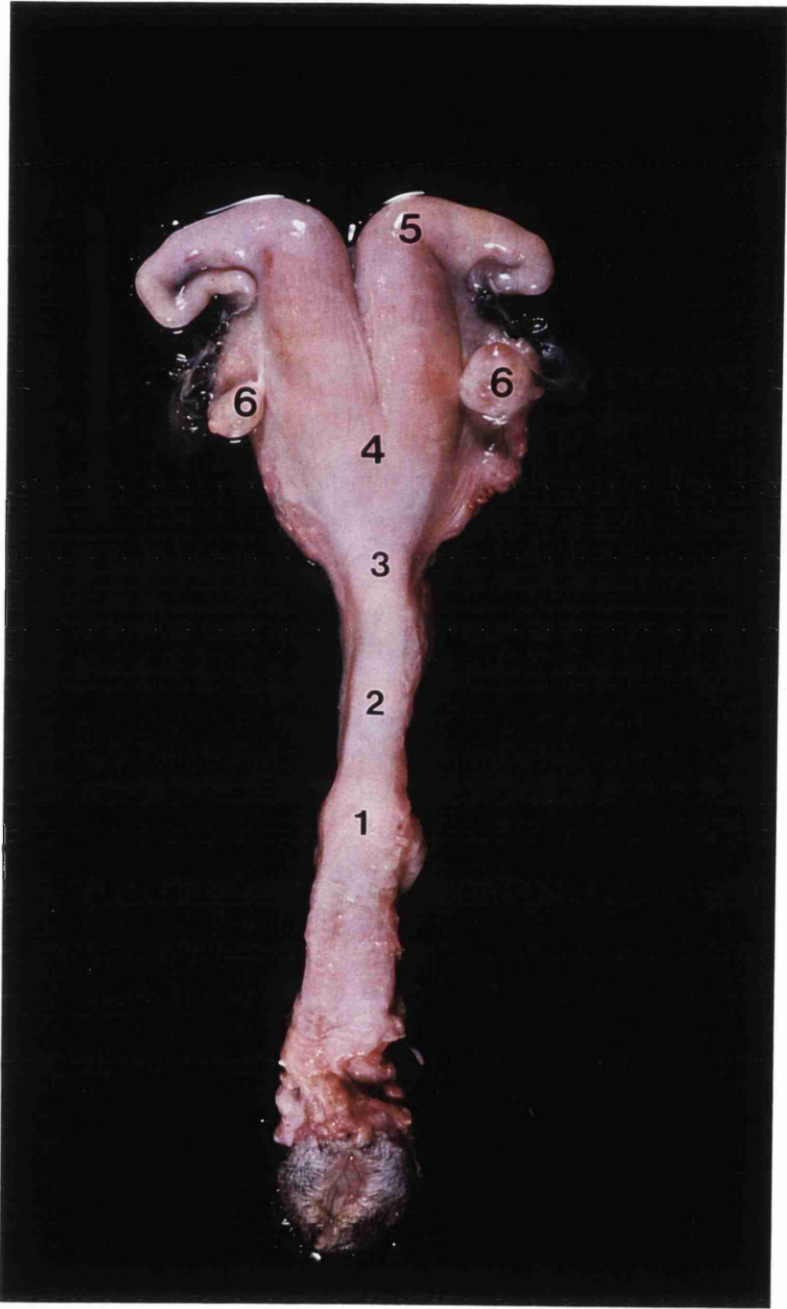
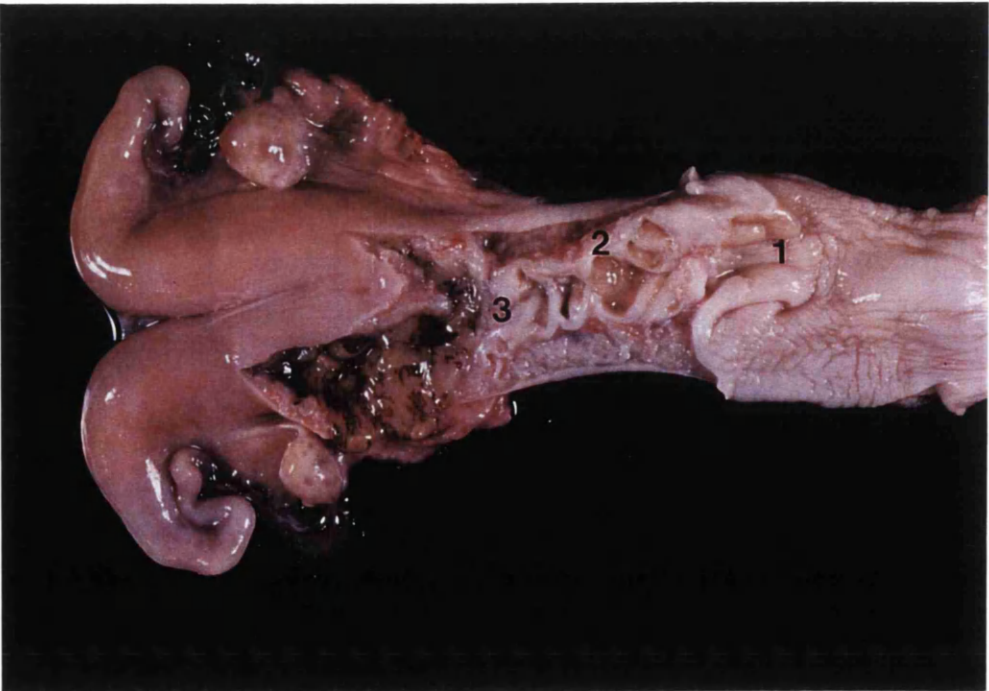
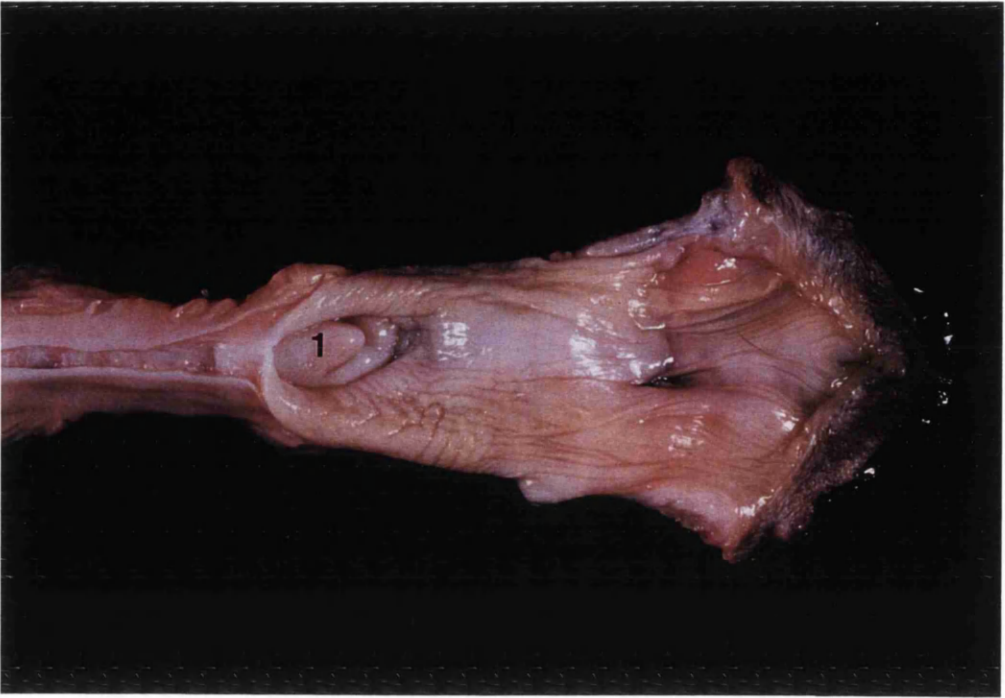


Plate 1.2

Gross anatomy of the ovine reproductive tract at the early luteal phase, showing the cervix uteri and the obstacles for catheterization.

Upper. Longitudinal section of the caudal part of the tract to show the external os (1) of the cervix uteri.

Lower. Longitudinal section of the ovine reproductive tract, showing the mucosal folds of the cervix. 1: external os, 2: mid cervix and 3: internal os



There have been a number of studies on the anatomy of the cervix aimed at the improvement of artificial insemination techniques. The early studies looked at the external os (Dun, 1955; Fukui and Roberts, 1978). Dun (1955) observed extreme variability in this structure within the Merino breed and attempted to classify the os types in order to improve the recognition of the opening of the cervical canal. He also tried to predict the success in cervical passage based on the external appearance of the cervix. It was concluded that pregnancy and parturition increase the size and complexity of the external os and therefore the identification of the cervical opening would be more difficult in aged ewes. Fukui and Roberts (1978) in similar studies, concluded that although the finding of the cervical orifice becomes more difficult within aged pluriparous ewes, the penetration of the cervical canal is easier. They proposed a method for non-surgical intrauterine insemination, based on the incomplete penetration of the cervical canal and the aid of CO₂ gas at high pressure to force the semen into the uterus.

The anatomy of the cervical canal was studied in detail using casts, these studies have shown that the cervical lumen has the shape of a row of cones out of alignment whose bases are cranial (Moré, 1984; Kristinsson and Wibdorf, 1985; Reinhold et al., 1987). Other techniques have been used, like fluoroscopy, ultrasound, xerography with Conray 400, Computer Axial Tomography and X-rays (Halbert et al., 1990a), and some of these have provided a good knowledge of the cervical canal in relation to AI. The cervical folds fit together closely, especially in the caudal part of the cervix, they are out of alignment and funnel shaped with the narrow opening oriented caudal (Halbert et al., 1990a). Reinhold et al. (1987) concluded that there were no anatomical variations between individual sheep in the portio vaginalis, orificium uteri externum or canal cervical, in relation to age or oestrous phase that could be exploited for the improvement of

transcervical AI techniques. More recent studies of the external os shape in cross breed ewes in relation to AI, also concluded that unfortunately this parameter is not related to internal parameters like mean cervical length, number of folds and width (Halbert et al., 1990a). Kristinsson and Wibdorf (1985) demonstrated the cervical lumen is not completely occluded by the folds, and suggested that a transcervical method is possible but has to be adapted to the peculiar anatomy of the ovine cervix. Halbert and colleagues from Guelph University have develop such a method based on their anatomical studies for different breeds (Halbert et al., 1990b; Buckrell et al., 1994).

The Guelph transcervical method of AI has been developed in Canada (Halbert et al., 1990c) but very poor results have been obtained, using the same technique, in countries like the UK (Mylne and Dingwall, 1993a; Mylne et al., 1993b) and Australia (Windsor et al., 1994). The problems in the UK have been embryonic loss and physical damage to the cervix and peri-cervical organs, like peri-cervical abscessation, metritis, and peritonitis associated with a track to a penetrated cervix.

A different approach was undertaken in this thesis, based on the pharmacological dilatation of the cervix with prostaglandin E₂ (PGE₂) to facilitate AI and ET. An effort was made to understand the physiological mechanisms of cervical ripening during parturition, that may give clues for the development of pharmacological regimes that could induce ripening during the cycle. This method may be an alternative or a complement to the Guelph and other similar methods.

1.2 MICROSCOPY OF THE CERVIX

The histology of the ovine cervix has been described in early studies (Cole and Miller, 1935; Restall, 1966; Restall and Lightfoot 1970; Hafez, 1973) and more recently reviewed (Moré, 1984; Dobson, 1988). In general the microscopic structure of the ovine cervix is similar to other species (Dobson, 1988). There are three different layers in transverse tissue sections: a) the endocervix, b) the fibromuscular wall and c) the serosa.

a) The endocervix

The external os of the cervix is an area of transition between the typical epithelium of the cervix (columnar) and the typical epithelium of the vagina (squamous). The lumen of the rest of the cervix is lined by a single layer of tall columnar cells. Three cell types can be differentiated, mucous secretory cells, tall ciliated cells and short peg-shaped cells; their nuclei are at different level giving a pseudostratified appearance. The epithelium shows regional variations, there are fewer mucous secreting cells in the cranial cervix near the internal os and the number of ciliated cells decreases caudally (Restall, 1966; Restall and Lightfoot, 1970; Hafez, 1973).

The epithelium undergoes similar cyclical changes to the rest of the reproductive tract, the secretory activity of the goblet cells peaks at proestrus (Restall, 1966). Two cell types have been identified using scanning electron microscopy at the early luteal phase, about 30% are ciliated and the rest are non-ciliated secretory cells. Before oestrus (days 15 and 16 of the cycle) the secretory cells further differentiate into two cell populations, the dilated secretory cells and the convex secretory cells (Wergin, 1979).

The subepithelial connective tissue consists of supporting cells, fibroblasts and fibrocytes, which are surrounded by ground substance, rich in fibroproteins and glycosaminoglycans (GAGs). This tissue is vascular

and white cells can be observed (Aughey et al., 1981).

The endocervix is arranged in large folds or cervical crypts, at the base of the crypts it is common to find stratification of the epithelium of 4-6 layers deep (Cole and Miller, 1935). The number of crypts is smaller during dioestrus (Restall, 1966) and anoestrus (Cole and Miller, 1935).

b) The fibromuscular wall

The cervix is now considered a separate organ from the uterus structurally as well as functionally by most authors, because the fibromuscular wall is composed mainly of fibrocollagenous tissue and some smooth muscle, whereas the uterus is predominantly muscular. Danford suggested these ideas for the first time in the late 1940s and early 1950s, demonstrating with histologic methods in women that the predominant component (85%) of the cervix, unlike the uterus is connective tissue (reviewed by Danforth, 1983). Similarly, the muscular content in the cervix is highest near the uterus (25%) and decreases caudally, being 16% in the middle segment and 6 % in the lower segment (Challis and Olson, 1988). Similar studies have not been done in sheep but studies in the rat confirm the fibrous nature of the cervix and the regional differences found in humans, suggesting that a common basic structure may exist within mammals (Koob and Ryan, 1980). The histology of the non-pregnant ovine cervix has been well documented, with studies of light (LM), scanning (SEM) and electron microscopy (TEM). The cervical wall consists of heavy, densely-packed collagen fibres interspersed with some fibrocytes and the occasional fibroblast (Ward, 1968; Aughey et al., 1981; Fosang et al., 1984; Owiny et al., 1987). There are very few elastic fibres (2-10 %) in the ovine cervix (Ward, 1968).

However, another group of authors have claimed that the ovine and human cervixes are predominantly muscular. Moré (1984) compares the smooth muscle of the ovine cervix with organs of propulsive activity such as

the uterus, the oesophagus, biliary pathways and the ureter, Hafez (1973) compares the cervix of primates with the iris diaphragm. In contrast, Aughey et al. (1983) claim that the muscular fibres form an incomplete muscularis in the ovine cervix and cannot have a sphincter-like function. There are different descriptions of the arrangement of the smooth muscle fibres. Raynaud (1973) describes the cervical musculature of the ewe divided into two layers, an inner layer composed of circular bundles and an outer layer composed of longitudinal bundles. Fosang et al, (1984) found bundles, running both longitudinal and transversely, present in the middle and deeper layers. Aughey et al. (1981), as mentioned earlier, describe an incomplete muscularis. Moré (1984) describes a more complicated organisation of the smooth muscle into five distinct layers.

c) The serosa

The peritoneum externally limits the cervix and consists of a thin layer of loose connective tissue covered by mesothelial cells (Moré, 1984). It does not merit further comments because it does not have much functional significance.

1.3 BIOCHEMICAL COMPOSITION OF THE CERVIX

The cells in the cervical connective tissue represent only 20% of its volume, thus the major components of the cervix are water and extracellular matrix (reviewed by Leppert and Yu, 1991). In sheep, several authors sustain that the rigidity of the cervix is due to its collagenous nature, collagen constitutes 50% of the dry matter in the non pregnant animal (Ward, 1968; Fitzpatrick, 1977; Fitzpatrick and Dobson, 1979). Similarly, in humans, chemical measurements of actomyosin have confirmed the histologic findings cited before that the cervix is predominantly connective tissue (reviewed by Leppert and Yu, 1991).

1.3.1 Extracellular matrix

In cell biology the ground substance of the connective tissue (see histology section) is often termed as extracellular matrix, which refers to an organised assembly of macromolecules located outside the cell. This matrix is produced locally by support cells, in the cervix the fibroblast/fibrocyte. The extracellular matrix is composed of two major macromolecules: (1) the fibrous proteins, of two functional types: structural, collagen and elastin, or adhesive, fibronectin and laminin, and (2) polysaccharide chains of the class named glycosaminoglycans (GAGs) normally associated to proteins in the form of proteoglycans (Woessner, 1993; Alberts et al., 1994a).

The extracellular matrix is a dynamic structure, subject to degradation by support cells and resident or infiltrated inflammatory cells. Two groups of proteinases are important in the remodelling of the extracellular matrix, the matrix metalloproteinases and the serine proteinases.

1.3.1i Fibrous proteins

Collagens

In all collagens, three polypeptide chains called α chains, are wound around one another in a rope-like helix, leaving relatively short non helical telopeptides on either side, this constitute a tropocollagen molecule. So far, about 25 genetically distinct types of α chains have been identified, each with distinct amino acid composition. A high content of the amino acids, proline and glycine, is typical of collagen, they are important for the formation and stability of the triple helix. So far, 15 types of triple stranded collagen (types I-XV) have been found, they differ in the α chain composition, strength of the bonds between the α chains, tissue distribution and supraorganization of the tropocollagens into: fibres (collagens type I, II, III, V and XI), fibre-associated (IX and XII) and networks (IV and VII) (Nimni, 1988; Alberts, et al., 1994a). In the reproductive tract, only a few types of collagen have been detected, but as newer tools for research become available other types may be found in this expanding family (Kokenyesi, 1991).

The ovine cervix is made up of the "classical" fibrillar collagens, mainly type I and III but also type V (Raynes et al., 1994). In other species (rats and humans) only types I and III of the fibrillar collagens have been reported and quantitative studies have shown that type I is the most abundant. In humans, type I constitutes about 2/3 of the total and 1/3 is type III collagen, this ratio is similar to that found in the skin (Uldbjerg, 1989; Kokenyesi, 1991). Tropocollagen molecules in collagens I and III, are arranged into the "quarter stagger array", that produces the characteristic cross-striation seen in electron micrographs. This structure is secured by the formation of "cross links" between the tropocollagen molecules (intermolecular cross-links), which are located between telopeptides and

triple helices. Cross links therefore, provide great tensile strength in the collagen fibres, keeping the tropocollagen fibres together and avoiding tearing (reviewed by Uldbjerg, 1989).

The mesh-forming type IV collagen has also been found in the human cervix and it is also expected to be found in sheep and other species. Type IV usually is present in the basement membranes, the tropocollagens do not form cross links in this type of collagen (reviewed by Uldbjerg, 1989 and Kokenyesi, 1991).

Elastic fibres

The presence of elastic fibres in the extracellular matrix is responsible for the elastic properties of a number of tissues like lung, dermis and large blood vessels (reviewed by Boyd et al., 1991; Rosenbloom et al., 1993; Alberts et al., 1994a), including the cervix (Leppert and Yu, 1991). Histological studies, have shown that there are very few elastic fibres (2-10 %) in the ovine cervix (Ward, 1968). Elastic fibres of the cervix and uterus are thinner, and may be easier to degrade than those of the lung or the aorta (Leppert and Yu, 1991).

Elastic fibres are composed of an amorphous component and a microfibrillar component. The protein elastin makes up the amorphous component and is responsible for the elastic properties of the fibres. There is a 0.95-2.45% of elastin in the cervix of primates (Leppert and Yu, 1991), which is similar to the skin (2-4%) and lower than large arteries (50%) (Rosenbloom et al., 1993).

Elastin is highly hydrophobic, this is consistent with its physical properties, the gene has been characterised in sheep and many other species. The biosynthetic precursor (tropoelastin) is released into the extracellular space, where it becomes strongly cross linked into a rubber-like network. The exact composition of the microfibrills is unknown, but they

contain several glycoproteins including fibrillin. They probably act as an organising scaffold in the formation of the elastin network.

Adhesive glycoproteins

These proteins have the ability to interact with cells and other macromolecules of the matrix, by coupling to receptors. The significance of this group therefore is not the provision of mechanical strength, but the alteration of the behaviour of cells in contact with them and the organisation of the matrix. The best studied is fibronectin involved in migration of cells, protease production and haemostasis/thrombosis (Alberts et al., 1994a). There are reports of the presence of fibronectin in the cervix, in the human and the rat (Leppi et al., 1982).

Laminin is a major component of the basement membrane and participates in attachment, migration, angiogenesis, protease production, tumour growth, metastasis and chronic inflammation (Alberts et al., 1994a).

1.3.1 ii Proteoglycans and Hyaluronic acid

Proteoglycans are special glycoproteins composed of a core protein and glycosaminoglycan (GAG) side-chains (chondroitin sulphate, dermatan sulphate, heparan sulphate, heparin and keratan sulphate). The old nomenclature was based on the GAG composition, the new nomenclature refers to the protein core composition and/or the function of the proteoglycan.

GAGs are sulphated polysaccharides composed of repeating disaccharide units (glucuronic acid, iduronic acid, N-acetyl galactosamine, N-acetylglucosamine and galactose). GAGs do not exist free of the protein core in the tissue with the exception of hyaluronic acid.

The mechanical significance of these substances is based on their ability to bind to collagen with variety of affinities, due to high sulphate

content they have a characteristic negative charge, this allows electrostatic interactions and made them highly hydrophilic. They have a role resisting compressive forces (reviewed by Christiansen et al., 1991).

The non-pregnant cervix contains similar proportion of GAGs (1% of the dry defatted tissue) as other connective tissues (reviewed by Uldbjerg, 1989). The most abundant proteoglycan in the cervix of all the species studied (sheep, human, guinea pig and rat) is decorin, also known as dermatan sulphate proteoglycan-II (DSPG-II), with a small (45 kDa) protein core and a single chain of either chondroitin sulphate or dermatan sulphate. Estimates of decorin as a percentage of the total of proteoglycans range from 50-80% in the non-pregnant cervix (reviewed by Dobson, 1988; Uldbjerg, 1989; Kokenyesi, 1991). This small proteoglycan is known to interact with collagen, there is microscopical and biochemical evidence of this (Uldbjerg, 1989). Medium size proteoglycans have not been detected by some authors and have been detected in variable proportion by others (reviewed by Dobson, 1988; Uldbjerg, 1989).

1.3.1 iii Degradation of the matrix by proteinases (endopeptidases): matrix metalloproteinases and serine proteinases.

Matrix metalloproteinases

Matrix metalloproteinases (MMPs) constitute the main proteolytic group capable of degrading all the components of the extracellular matrix at a physiological pH. This family has many other names that define some of their characteristics as a group, like neutral proteases, collagenase gene family and matrixins (reviewed by Nagase, 1991; Woessner, 1991; Matrisian, 1992; Birkedal-Hansen et al., 1993; Woessner, 1994).

The interest in MMP is increasing because they seem to be involved

in multiple biological processes requiring remodelling of the connective tissue. Apart from cervical ripening at parturition, they are also involved in other reproductive (ovulation, embryogenesis, implantation and postpartum uterine involution), physiological events (wound healing and bone resorption), and several pathological processes (rheumatoid arthritis, periodontal disease, metastasis and inflammation in general) (Woessner, 1994). MMPs are secreted from connective tissue cells or inflammatory cells as inactive zymogens (proenzymes). Most matrixins are not stored in the cell, are only synthesised upon demand and stimulated by a number of factors (PGE₂, interleukins, steroids, growth factors, oncogenes etc.) (Birkedal-Hansen et al., 1993). The MMPs of PMLs are exceptions, and they are stored in granules (Doherty and Janusz, 1994). The extracellular activity of MMPs is regulated in a complex fashion. They are activated when cleaved by other proteinases in a cascade. The PML collagenase is also an exception in this, and is activated as part of the PML activation mechanisms (Doherty and Janusz, 1994). In the active form metalloproteinases are regulated by inhibitors present in plasma (α -macroglobulin) or in the extracellular matrix by tissue inhibitors of the metalloproteinases (TIMPs). These are generally secreted by the same cell as the metalloproteinases and form inactive complexes with these enzymes. Three different TIMPs (TIMP-1, TIMP-2 and TIMP-3) have been identified and cloned in women (Woessner, 1991; Woessner, 1994). So far only ovine TIMP-1 has been cloned (Smith et al., 1994).

Up to the present moment, 12 members of the MMP family have been identified and well characterised from cDNA sequencing, mainly in women (Woessner, 1994). They belong to three major subclasses according to their main matrix substrate and structural homology, namely collagenases, gelatinases and stromelysins (Nagase, 1991; Woessner, 1991; Matrisian, 1992).

Collagenases

Two collagenases have been cloned in women, interstitial collagenase (MMP-1) and PML collagenase (MMP-8). So far none of them have been characterised at the molecular level in sheep, although there is evidence of collagenase activity in sheep (Ellwood et al., 1981; Raynes et al., 1988). Ovine MMP-1 appears to cross react with the human enzyme since its activity has been detected in ovine tissues using human probes (Salamonsen et al., 1993). MMP-8 has only been studied in women, but MMP-1 has been sequenced in the bovine and other species (Sudbeck et al., 1992; Woessner, 1994). Studies of bovine MMP-1 have shown poor cross-reactivity with other species but similar sequence and cleavage point (Sudbeck et al., 1992).

MMP-1 is the collagenase found in fibroblasts and macrophages and was the first MMP to be described. It degrades the interstitial collagens of the cervix and also type II, in the order: III>I>II (Docherty and Murphy, 1990). It cleaves in the helical portion of the collagen molecule specifically, breaking the 3 α chains at a single site and generates two 3/4 (TCA) and 1/4 fragments (TCB). The specific degraded products of collagenase (TCA and TCB) denature and unfold at body temperature (gelatine) and are susceptible to degradation by gelatinases and non-specific neutral proteases.

PML collagenase (MMP-8) is 57% homologous to the amino acid sequence of MMP-1 and heavier in its molecular weight. It can also degrade interstitial collagens with the same specificity as the MMP-1 but its rate of hydrolysis is different being in the order: I>II>III (Doherty and Murphy, 1990).

Serine proteinases

The serine proteinases group, includes plasminogen activators, plasmin, elastases, thrombin, trypsin, chymotrypsin, and cathepsins G and E. Some of these enzymes participate in the remodelling of the matrix by direct degradation of several matrix components or by participating in the activation of the MMPs. Their actions are regulated by specific inhibitors (Masure and Opdenakker, 1989; Caughey, 1994; Alberts et al., 1994a).

Leukocyte elastase

Little is known about leukocyte elastase in sheep, but recent studies have reported the isolation of a similar enzyme to the human leukocyte elastase (HLE) from the ovine PML (Junger et al., 1992). Interspecies variability has been reported in the levels of activity of leukocyte elastase in PMLs. Rabbits, for example have twice the activity of human PMLs, whereas other species have only one third (equine and porcine) or 50 times less (bovine) (Styrt, 1989; Brown and Roth, 1991).

Human leukocyte elastase (HLE) can degrade elastin, collagens type III and type IV and other components of the matrix (proteoglycans, fibronectin and fibrin) (Masure and Opdenakker, 1989; reviewed by Uldbjerg, 1989). HLE degrades the telopeptides of collagen, thus removing intermolecular cross links which are important for the stability of the collagen fibre. This action may facilitate access of collagenase to its substrate, increasing its cleavage rate (reviewed by Uldbjerg, 1989). Another synergistic action of HLE with collagenase is that it can activate stromelysin-1 (MMP-3), which is the activator of proMMP-1 (Leppert, 1992). Leukocyte elastase and other serine proteinases, like trypsin and α -chymotrypsin, can upregulate the activity of several MMPs by blocking the inhibitory action of TIMPs (reviewed by Docherty and Murphy, 1990).

The three most important HLE inhibitors or antiproteinases are: α 1 proteinase inhibitor (A1PI, formerly called antitrypsin), α 2-macroglobulin, and secretory leukoproteinase inhibitor (SLPI). Secretory leukoprotease inhibitor (SLPI) is found in mucous secretions (seminal plasma, cervical mucus, bronchial and nasal secretions) and interstitial fluids. It has a role in the protection of mucous membranes during inflammation (Leff and Repine, 1993; Uldbjerg, 1989). But the most potent inhibitor is probably A1PI, which forms a complex with elastase, inactivating it irreversibly. Despite this tight control, tissue damage occurs at inflammatory sites, since the three inhibitors are susceptible to oxidative inactivation.

Summarising, the triple helix of collagen is resistant to most enzymes and therefore the action of collagenase (MMP-1 or MMP-8) is considered essential and rate-limiting in the degradation of collagen. But it is also recognised that collagenolysis is the result of the action of a group of enzymes in a cascade. Stromelysins may facilitate degradation of collagen by removing the surrounding proteoglycans. PML elastase can activate the proMMP3, and MMP3 in turn activates proMMP1 (Leppert, 1992). Additionally serine proteinases from white cells also act synergistically by disrupting the matrix with their degradative action on proteoglycans, cross links and elastin. Gelatinases further degrade the products of collagenase. Cathepsins act intracellularly degrading collagen that has been phagocytosed and partially degraded.

1.4. CERVICAL PHYSIOLOGY DURING THE CYCLE AND PREGNANCY.

The cervix adapts to its different roles (sperm transport, protection of the uterus against infection and delivery) with changes in the composition of the mucus produced and of its own structure. During oestrus, there is an increase in vascularity, accompanied by congestional oedema, relaxation in the cervical muscular tone and opening of the cervical canal. At oestrus the mucus production increases and it acquires special characteristics which are permissive to sperm transport; mucus becomes thinner, the sodium chloride content and alkalinity increase, whereas the cell content decreases. The oestrous mucus, due to a high content of sodium chloride, when spread on glass slides crystallises in a pattern that resembles a fern, the phenomenon is known as fernlike crystallisation or arborisation (reviewed by El-Banna and Hafez, 1972). At other times, under the influence of progesterone, the cervical canal remains closed to protect the uterus from infection and only a small amount of highly viscous mucus is present in the canal. During gestation, the cervix grows and secretes large masses of viscous mucus, which forms a plug that occludes the canal, acting as a barrier to bacteria and sperm (Hafez, 1973). During the second half of pregnancy, the ovine cervix experiences gross anatomical changes in consistency, size and shape that are known as softening that culminate with “ripening” during full term and the onset of labour, that occurs over a period of five hours or less (Ward, 1968; Owiny, 1986; Fitzpatrick, 1977).

During mid (crown-rump length of the fetuses: 13-19.5 cm) and late pregnancy (crown-rump length of the fetuses: 23-39 cm) the cervix experiences softening, becoming oedematous and floppy and there is a small increase in compliance and a marked increase in size (Aughey et al., 1981; Owiny et al., 1994). The cervical mucus plug is dissolved just prior to labour (Aughey, personal communication). In the sheep most of the

softening and increase in compliance occurs 12 hours before the onset of labour (Fitzpatrick and Liggins, 1980). Similar changes, but over a longer period of time occur in other species (human: Calder, 1981; rat: Hollinsworth, 1981). Postpartum the cervix experiences a rapid involution and returns to its original shape (Datta et al., 1968; Lo Stumbo and Pellegrini, 1970; Fosang et al., 1984). Of all these functions, ripening at prepartum has been the most extensively studied.

Cervical ripening during labour has been extensively reviewed on its own in several books and review articles (Fitzpatrick, 1977; Danforth, 1983; Dobson, 1988; Uldbjerg, 1989; Uldbjerg and Ulmsten, 1990; Huzar, 1991; Hayashi, 1993) or as a chapter of parturition reviews (Challis and Olson, 1988; Taverne, 1992; Porter, 1993; Maltier et al., 1993a; Challis and Lye, 1994; Kelly, 1994). Most of the information refers to human physiology, because the unripe cervix is a frequent problem in obstetrics and premature dilatation increases the risk of uterine infection and possible loss of the fetus. The pregnant and periparturient ovine cervix has been more extensively studied than in other domestic or experimental species from the 1970s (Ward, 1968; Fitzpatrick and Dobson, 1979; Owiny, 1986; Owiny and Fitzpatrick, 1992). The ewe was adopted as a experimental model for a number of reasons, the toughness and collagenous nature of the cervix, the well known endocrinology of birth (Thorburn and Rice, 1990). Like humans, sheep is a placental-dependent species for the production of progesterone at late pregnancy (Liggins, 1983) and there is a clinical condition when the cervix fails to ripen (ringwomb) (Ward, 1968). However the endocrinology of the guinea pig at parturition appears to be more similar to humans than than ovine endocrinology and this laboratory species is gaining more importance as an experimental model for cervical ripening, in recent years (Rajabi et al., 1990; Chwalisz, 1994). Extensive studies have also been done in the mouse, rat, rabbit and pig (Dobson, 1988).

1. 5 PHYSIOLOGICAL CERVICAL RIPENING OF THE PERIPARTURIENT CERVIX: MICROSCOPICAL, MECHANICAL AND BIOCHEMICAL CHANGES

1.5.1 Microscopy

The gross anatomical changes of cervical ripening are correlated with microscopical changes in the connective tissue in sheep with separation of collagen fibres that have been observed with light, transmission and scanning electron microscopy (LM: Ward, 1968; Aughey et al., 1981 and 1983, TEM: Fitzpatrick, 1977; Parry and Ellwood, 1981; Aughey et al., 1981 and 1983; Fosang et al., 1984; Owiny, 1986, SEM: Owiny et al., 1987). Similar changes have been observed in other species (women: Danforth, 1960; Minamoto et al, 1987; Yoshida and Manabe, 1990; rats, mouse, pig, rabbit: Dobson, 1988; guinea pigs: Rajabi et al., 1991b). There is an infiltration of PML (Junqueira et al., 1987) and other inflammatory cells and therefore changes have been compared to an inflammatory response (Liggins, 1981) and with other reproductive or pathological processes of rapid tissue remodelling and collagenolysis (Luque and Montes, 1989). In sheep, microscopical changes with evidence of tissue breakdown have been observed during the last third of pregnancy, approximately from about day 100-120 of pregnancy (determined by crown-rump length of the fetuses) (Aughey et al., 1981) and from day 140 of gestation onwards (Fosang et al, 1984).

Microscopical observations during the postpartum involution suggest that this process consists of the reorganisation of the collagen and restoration of the dense connective tissue and begins 18 hour postpartum (Fosang et al, 1984).

1.5.2 Mechanical properties of the cervix

The mechanical properties of the cervix (compliance, extendibility and stretch modulus) have been measured in sheep and other species (women, rat, goat and guinea pig) during the physiological ripening associated with labour (reviewed by Dobson, 1988).

Studies on the mechanical properties of the ovine cervix around spontaneous or induced parturition show a very abrupt increase in compliance a few hours before the appearance of uterine contractions, within 36 hours of labour (Fitzpatrick, 1977; Fitzpatrick and Dobson, 1981; Ledger et al., 1985; Owiny et al., 1987). Studies throughout pregnancy, have shown a decrease in the stretch modulus in early pregnant sheep (30-50 days of gestation) that was significantly different from non pregnant sheep, indicating the beginning of cervical softening. Softening gradually increased from day 30 and day 140 of gestation, with a marked significant increase between term (day 140-144) and the onset of labour (145-147 days). No further distension occurs between labour and the delivery of the lamb (Owiny et al., 1991). There is therefore good correlation between the timing of microscopical changes and changes in the mechanical properties of the periparturient ovine cervix.

1.5.3 Biochemical changes in the extracellular matrix

Biochemical studies have corroborated the idea that changes in the connective tissue are responsible for cervical ripening at term. These studies are more complete in women although extensive studies have also been done in sheep, rats and guinea pigs. The matter is not fully clarified and there are several theories and differences between laboratories and species. Changes in the biosynthesis and metabolism of collagen and proteoglycans and enzymatic degradation of collagen seem to be the key points.

Summarising, the two main mechanisms of cervical ripening could be: 1) Enzymatic degradation of key constituents of the extracellular matrix, involving MMP-1 or MMP-8 and leukocyte elastase. 2) Changes in the amount and relative composition of the proteoglycans and GAGs which destabilise collagen.

In the guinea pigs and rabbits there is evidence of the enzymatic degradation of collagen, which is restricted to the activity of fibroblast collagenase (MMP-1) (Rajabi et al., 1990; Imada et al., 1994). But in human studies, there is evidence accumulating, that involves PML degranulation with the release of PML collagenase (MMP-8) and leukocyte elastase (Doherty and Janusz, 1994) in the degradation of collagen and other components of the matrix (Uldberg (thesis) 1989; Rath et al., 1994; Morrison et al., 1994).

In the ovine cervix, collagenase activity has been detected with assays that do not differentiate MMP-1 from MMP-8 and its possible role in cervical ripening is still unclear. There is evidence for an increased activity of collagenase at term, using an activity based assay (Ellwood et al., 1981). But other collagenase assays have not been able to detect the increase: such as the less sensitive hydroxyproline assay (Fitzpatrick and Dobson, 1981) or another activity based assay (Raynes et al., 1988a). Similarly, in rat cervical tissue, attempts to extract collagenase obtained very low levels (unpublished observations), and it was suggested by some authors that species variability may exist in the mechanisms of ripening and that changes in GAGs and proteoglycans could be more important in species like the rat and the sheep (Woessner and Kokenyesi, 1991).

Initially there were technical difficulties in demonstrating an increase of collagenase activity before parturition in the guinea pig (Rath et al., 1989) and in women, but a year later the role of collagenase prepartum was demonstrated in both species (women: Osmers et al., 1990; guinea pig:

Rajabi et al., 1990). It is not clear, therefore, if the failure to detect an increase in collagenase activity in sheep and rats reflects true physiological differences in the mechanisms of cervical ripening, technical problems or species differences in the levels of collagenase. A common mechanism with the involvement of collagenase in sheep and rats may be supported by: (1) an increase in collagenase activity has been demonstrated, inducing labour in rats with RU486 (Ikuta et al., 1991), (2) the method used by Rajabi and colleagues in women and guinea pigs, is 3 times more sensitive than the one used by Raynes and colleagues in sheep (Rajabi et al., 1990) and (3) Osmer and colleagues (1991b) suggest that there is a limited temporal window of collagenase activity, which may be even more critical in the sheep, due to the speed of the events during cervical ripening compared to women.

The regulation of MMP has also been studied in relation to cervical ripening, which further supports the enzymatic theory. Regulation can be achieved in 3 ways: a) control of gene expression of MMP, b) extracellular activation of proenzyme to enzyme and c) by tissue inhibitors of the MMPs (TIMPs) (Woessner, 1991). Measurements of TIMP by ELISA showed elevated levels at the end of pregnancy in women (37-40 weeks) at a time when the collagen content decreased substantially (Clark et al., 1994). The authors suggest that the role of TIMP may be preventive against massive uncontrolled degradation by metalloproteinases. Similar findings were reported when studying cultured cervical tissue in the sheep. The amounts released of the collagenase inhibitor was greatest from tissue at peripartum (145-146 days, term: 147 days). However, these results were interpreted as conflicting with the collagen degradation theory for cervical ripening in sheep, because a substantial increase in collagenase activity was not detected (Raynes et al., 1988b). In women, although collagenase inhibitors increased significantly at term, collagenase increased much more (23

times) than the sum of its inhibitors TIMP-1 and α 2-macroglobulin (twofold) suggesting a net breakdown of the tissue. Similar imbalance between collagenase and its inhibitors had been observed by one of the authors in osteoarthritis (Rechenberger and Woessner, 1993). *In vitro* studies in the rabbit, have shown that progesterone may keep the cervix closed during pregnancy by increasing the production of TIMP-1 and TIMP-2; these inhibit the enzymes MMP-1 and MMP-3 respectively (Imada et al., 1994).

During pregnancy (100 days, 140 days and at term), the softening of the ovine cervix is accompanied by an overall reduction of the collagen content (Fosang and Handley, 1988). Similar results have been found in women, collagen content decreased from 10 weeks of pregnancy and in periparturient women is between 30-50% of that in the non-pregnant control. The extractability of collagen increased, from 40% in non pregnant cervixes to 95% in ripened cervixes, and shows new collagen with fewer cross links (Uldberg (thesis) 1989). The activity of the collagen-breaking enzymes collagenase and leukocyte elastase, originated from chemoattracted PMLs could explain these changes in humans (Osmers et al., 1992; Rath et al., 1994). It is also possible that the source of collagenase is the fibroblast, since mRNA for MMP-1 has been detected in cell cultures of the late pregnant guinea pig cervix (Rajabi et al., 1990). There is also an increase in the water content, that can be explained by increased vascularity and changes in GAGs (Dobson, 1988; Uldbjerg et al., 1983).

Although there is strong evidence for the enzymatic theory in women and the guinea pig, the importance of the proteoglycans is not ignored in these species (Osmers et al., 1993; Rechberger and Woessner, 1993). The changes in the proteoglycans are not fully agreed, and it is unclear if this is due to assays or species differences. Some authors report an increase in the synthesis of proteoglycans in general (Fosang and Handley, 1988),

others report changes in the proportion of the different types of GAGs, with an increase of hyaluronic acid whereas dermatan sulphate, the most abundant GAG, decreases; this has been found in sheep and in most studies from other species (reviewed by Dobson, 1988). The increase in hyaluronic acid may act by attracting water, and the decrease in dermatan sulphate proteoglycan may destabilize collagen and fibronectin which binds with high affinity to dermatan sulphate (Christiansen et al., 1991; Osmer et al., 1993). Chondroitin sulphate is unchanged during late pregnancy in the ovine species (Anderson et al., 1991), but an increase has been detected in humans. Unlike dermatan sulphate, chondroitin sulphate has a weak affinity for collagen and fibronectin and therefore an increase destabilises the extracellular matrix (Osmer et al., 1993). Heparan sulphate increases in sheep and women during late pregnancy, possibly acting on blood vessels and increasing the vascularisation (Anderson et al., 1991; Osmer et al., 1993). Other authors have reported an increase in the small dermatan sulphate decorin (DSPG-II), which could act by separating collagen fibres occupying more space between them. They postulated that the high solubility of the collagen is not due to degradation by collagenase or lack of cross linking but to dispersion of collagen fibres. Elastin could fit in this theory as a "memory" of the initial shape to be restored postpartum (rat: Woessner and Kokenyesi, 1991).

1.5.4 The possible role of the smooth muscle in cervical functions

The role and the function of the smooth muscle in cervical ripening and other cervical functions remains unclear. Most of the literature is of the opinion that the cervix consists mainly of connective tissue (see previous histology and biochemistry sections, pages 10 and 12) and that cervical ripening is an active process, involving remodelling of the connective tissue

(section: 1.3, page 12). But some authors contend that the cervical musculature is sufficiently developed for it to show spontaneous contractility (histology section). Based on that view, some studies have shown spontaneous and drug-induced contractility during the cycle and in late pregnancy, but others consider the role of this contractility insignificant when compared to the uterus proper (reviewed by Challis and Olson, 1988; Hillier, 1990; Rousseau and Ménézo, 1993). There is also disagreement as to whether or not hypertrophy of the smooth muscle occurs during cervical ripening in late pregnancy (sheep: Aughey et al., 1983; human; Minamoto et al., 1987; human; Junqueira et al., 1980).

Cervical motility has been observed in the smooth muscle during the cycle (Garcia Villar et al., 1982) and during pregnancy in sheep (Garcia Villar et al., 1984a). The smooth muscle of the cervix is responsive to drugs *in vitro* and *in vivo*, with either contractions or relaxation. Prostaglandins in the cervical muscle, unlike in the uterus proper, tend to elicit relaxation (humans: Najak and Hillier, 1970; Hillier, 1990). In contrast, oxytocin induces contractions (Garcia-Villar et al., 1982), this effect unlike the uterus does not require oestrogen priming (Garcia-Villar et al., 1984b). Other studies, however, have pointed out that the ability of the cervix to contract, compared to that of the uterus, is negligible (human: Najak and Hillier, 1970).

In the ewe, two phased cycles of spontaneous contractility have been observed during the cycle, irregular activity and regular activity, only the latter occludes the cervix completely. During oestrus the irregular activity, which does not occlude the cervix, was more frequent than during the luteal phase (review Rousseau and Ménézo, 1993). Forty eight hours before lambing, a phase of relative inhibition of electromyographic (EMG) activity occurred both in the cervix and the uterus and lasted for 17 hours; it has been suggested that the increase in cervical compliance may start

during this phase of inhibition. Thereafter EMG activity returned and increased over the whole genital tract (reviewed by Taverne, 1992). An alternative interpretation is that unchanged contractility before and during lambing indicates that relaxation of the musculature is not responsible for cervical compliance (reviewed by Challis and Olson, 1988).

1.6 PHYSIOLOGICAL CERVICAL RIPENING AT TERM: THE ROLE OF PGE_2 AS PART OF THE ENDOCRINE AND PARACRINE REGULATION

It is difficult to separate the effects of the different hormones on the uterus and the cervix in late pregnancy and during parturition. Both organs are exposed to a similar endocrinological environment during this period, but they respond differently. Microscopical, mechanical and biochemical changes in the cervix, indicating softening and tissue break down, have been noticed at preterm (day 140) in sheep (see previous sections). But the maximal changes occur very rapidly within 36 hours prior to labour and coinciding with dramatic changes in plasma concentrations of progesterone, oestrogen and $\text{PGF}_{2\alpha}$ at 24 hours before the onset of labour (Fitzpatrick and Liggins, 1980; Owiny, 1986). There is a clear direct correlation between a drop in cervical compliance with falling levels of progesterone and rising levels of 17β oestradiol and prostaglandin $\text{F}_{2\alpha}$ (Fitzpatrick and Liggins, 1980), suggesting an endocrinological regulation by these hormones of cervical ripening.

A causal role for prostaglandins in ovine parturition has been demonstrated. The administration of the PG synthetase inhibitor meclofenamic acid to late pregnant sheep prevented cervical ripening and uterine contractions despite steroid changes, delivery took place when the inhibitor was withdrawn (Mitchell and Flint, 1978). On the other hand, cervical tissue from late pregnant sheep can produce prostaglandins at

high rates *in vitro* and there is evidence of an increase in production at delivery (mainly PGE₂ and PGF_{1α}). Studies *in vivo* also showed high levels of PGE₂ and 6-oxo-PGF_{1α} in cervical venous plasma 6 hours before delivery or at delivery respectively. These studies suggested a local regulation of cervical ripening by PGE₂ and other prostaglandins (Ellwood et al., 1981). Studies from other species have shown that oxytocin and relaxin may also be involved in cervical regulation by either endocrine or paracrine mechanisms (reviewed by Challis and Olson, 1988; Dobson, 1988; Hillier, 1990).

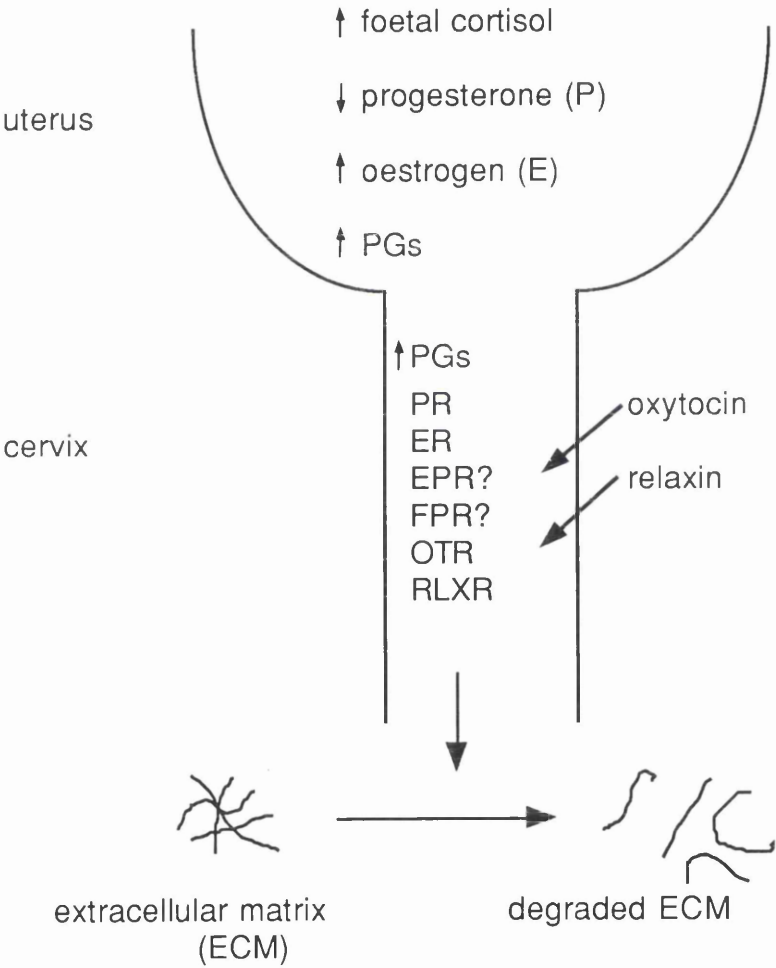


Figure 1.0: Endocrinology of cervical ripening

1.6.1 Decrease in the progesterone/oestrogen ratio (P4/E2 ratio)

1.6.1 i Progesterone withdrawal

In sheep, the high plasma levels of progesterone observed in the second half of pregnancy are due to placental production. Progesterone withdrawal occurs very rapidly, one day before the onset of labour (Chamley et al., 1973). This drop in progesterone has been attributed to the catalytic activity of 17 α -hydroxylase in the placenta converting progesterone into androgens which are subsequently converted to oestrogens (reviewed by Liggins, 1983). This mechanism produces a rapid shift in the P4/E2 ratio that occurs within hours in the sheep, faster than in *corpus luteum*- dependent species like the rat, where it is a matter of days rather than hours (Maltier, 1993a).

The source of progesterone is also the placenta in humans and the guinea pig, but the peripheral levels of progesterone remain unchanged before the onset of labour in these species. Therefore it was proposed that local concentrations of progesterone may drop in the uterus of these species (Challis and Olson, 1988). There is local production of steroids inside the uterus by fetal membranes in humans and other primates. In sheep steroidogenic enzymes are present in the fetal membranes too, suggesting a potential for a paracrine effect of steroids but it is not clear if systemic or paracrine effects are more important (Challis and Lye 1994).

The administration of antiprogestagens, acting at the receptor level, induce cervical ripening in all species studied supporting a paracrine regulation. Antiprogestagens induced full labour only in those species with

physiological progesterone withdrawal at term (rats, rabbits, sheep, pigs and cows), but induced cervical ripening in those species without a fall in peripheral levels of progesterone (guinea pigs, non-human primates and humans) (reviewed by Chwalisz, 1994). In women the P4/E2 ratio decreases by 40% locally in the uterus in the last few weeks, but this type of information is not available for the cervix (Maltier et al., 1993a). Other authors have proposed that a loss of progesterone nuclear receptors (PRn) occurs in the rat towards the end of pregnancy and human myometrial receptors are also very low at parturition (Giannopoulos and Tulchinsky, 1979; Khan-Dawood and Dawood, 1984). Similarly in the pig, progesterone nuclear receptors (PRn) in the cervix and myometrium, increased significantly at mid pregnancy and declined afterwards at the same time as the drop in plasma progesterone (Thilander et al., 1990). In the cow, parturition starts with already fallen levels of progesterone and the levels of myometrial progesterone receptors do not drop at parturition (Klauke and Hoffman, 1992). In sheep, parturition starts with declining levels of progesterone and myometrial receptors levels are very low (Klauke and Hoffman, 1992). These works suggest that in cattle removal of progesterone from the bloodstream occurs whereas in humans a similar effect may be achieved by a loss of progesterone receptors. In sheep and pigs both mechanisms may be important.

There is a positive correlation between plasma progesterone and softening of the cervix, measured by mechanical parameters, suggesting that progesterone withdrawal facilitates ripening (Fitzpatrick and Liggins, 1980; Owiny, 1986). But the importance of progesterone withdrawal as a prerequisite for cervical ripening is not completely clear in sheep. Massive doses of progesterone (200 mg/day) administered to sheep at parturition, inhibited contractions but were unable to inhibit the increase in cervical compliance in sheep (Stys et al., 1980). Others have found that cervical

ripening was blocked by the administration of high doses of progesterone (150 mg/day) at late pregnancy, in the presence of uterine contractions (Challis and Lye, 1994). Using lower and more physiological doses of progesterone (100 mg/day), labour progressed normally both in terms of cervical ripening and uterine contractions (Challis and Lye, 1994). Studies with the inhibitors of progesterone (epostane) and prostaglandin synthesis (mefenamic acid) in sheep, suggested that progesterone withdrawal was capable of inducing cervical ripening in the absence of an oestrogen surge but was dependent on prostaglandin synthesis (Ledger et al., 1985). Unlike the sheep, the effect of progesterone withdrawal does not seem to be mediated by prostaglandins in guinea pigs, man or rats. Inhibitors of prostaglandin synthesis did not abolish the effect of progesterone antagonists on the cervix. The administration of progesterone antagonists did not affect the synthesis of prostaglandins by cervical tissue (reviewed by Chwalisz et al., 1994).

Early studies in sheep showed that progesterone had an inhibitory effect on the production of collagenase by the cervix *in vitro* (Ellwood et al., 1981). In the guinea pig procollagenase (proMMP-1) gene expression seemed to be regulated by progesterone and oestrogen but results were unclear (Rajabi et al., 1990). In rabbit cervical fibroblasts, physiological doses of progesterone upregulated two inhibitors of metalloproteinases (TIMP-1 and TIMP-2) (Imada et al., 1994) and also downregulated two metalloproteinases, namely pro-interstitial collagenase (pro-MMP-1) and pro-stromelysin (pro-MMP-3) respectively (Sato et al., 1991). *In vitro* studies in human placenta (choriodecidua) obtained at term or after labour, have shown downregulation of IL-8 production by progesterone and upregulation by progesterone withdrawal with mifepristone (Kelly et al., 1992). These authors suggest the following mechanism of parturition, cervical softening and uterine contractions occur as a result of local

progesterone withdrawal that was suppressing IL-8 and collagenolysis by PMLs during pregnancy. Studies of this type are not available in the sheep, but similarly progesterone withdrawal is followed by a PML invasion of the endometrium (Staples et al., 1983). In rabbits progesterone also suppresses IL-8 production by uterine cervical fibroblasts (Ito et al., 1994).

Summarising, studies *in vitro* in a wide range of species (sheep, rabbit, guinea pig and humans) suggest that progesterone may have a negative effect on cervical ripening by regulating the degradation of the extracellular matrix. Progesterone could downregulate the gene expression and activity of matrix metalloproteinases by fibroblasts. Progesterone could also act as anti-inflammatory agent that suppresses the influx and activation (degranulation and release of proteinases) of PMLs, inhibiting the production of IL-8, for example.

1.6.1 ii Oestrogen surge

A very sharp peak of plasma oestrogens occurs 24 hours before labour in sheep (Challis, 1971), oestrone (E1) was produced in larger quantities than oestradiol-17 β (E2) (Chamley, et al., 1973). In other species the rise of E2 occurs more gradually within 5-6 weeks prelabour (Challis and Olson, 1988; Maltier et al., 1993a).

In the sheep, which is a "placental 17 α -hydroxylase (P-450_{17 α}) present species", fetal cortisol and placental progesterone are the main precursors of the synthesis of oestrogen by the placenta at parturition. Human is a "placental 17 α -hydroxylase deprived species", and different main pathways of oestrogen production are used, the placenta imports dehydroepiandrosterone sulphate (DHAS) from maternal and fetal adrenal origin to produce oestradiol (Liggins, 1981; Robel, 1993). This alternative pathway may be present in sheep, contributing in a "fail-safe" mechanism (Challis and Olson, 1988).

Physiological concentrations of DHAS may contribute to cervical ripening in women, since they increase in the cervix with gestational age and are higher in women with ripe cervixes, compared to unripe cervixes. It has been suggested that this androgen may be more effective than oestrogen in ripening the cervix. DHAS stimulated collagenase and other MMPs in pregnant rabbit uterine cervix in culture (reviewed by Hillier, 1991), which is also a P-450_{17 α} deprived species (Liggins, 1981). DHA may be involved in a paracrine manner in inflammation (Kelly, 1994).

In humans oestrogen can be synthesised in fetal membranes, suggesting a possible paracrine regulation of the uterus and cervix (Challis and Olson, 1988). Oestrogen receptors (ER) have been shown first in the rat myometrium through pregnancy, they decreased during pregnancy in both cytosolic and nuclear fractions (>300 to >100 fmol/mg protein) and sharply increased (from >100 to 200 fmol/mg protein) with the onset of labour (Alexandrova and Soloff, 1980). These results are explained by progesterone downregulation of ERs during pregnancy and upregulation by the increased E2/P4 ratio before labour, since oestrogens upregulate ERs (Alexandrova and Soloff, 1980). Similarly, ER mRNA increases in human uterine tissues during labour (Mitchell et al., 1993) and adrenalectomy in late pregnant sheep, which prevents the increased E2/P4 ratio, decreased markedly the mRNA expression of ER (Wu et al., 1993). But so far, only low levels of oestrogen receptors have been reported during labour in the ovine myometrium (15.3 fmol/mg protein) and a gradual decrease in the receptor concentration from the first trimester to parturition in cows (95.1 to 4.5 fmol/mg protein) (Klauke and Hoffman, 1992). In the porcine cervix and myometrium both cytosolic (ERc) and nuclear oestradiol (ERn) receptors increased in late pregnancy and were maximal at parturition. The number of ERc and ERn during parturition were 2000 and 60000 sites/cell in the cervix and reached levels of 4000 and

40000 sites/cell in the myometrium respectively (Thilander et al., 1990). The authors found difficulty in explaining the role of ER in the softening of the cervix at labour because they had previously concluded that ER mediates the constriction of the cervix during oestrus in the sow. The concentration of ER α and ER β in the porcine cervix at oestrus was about 7600 and 1200 sites per cell respectively (Stanchev et al., 1984).

Oestradiol appears to promote cervical ripening in most species but at times inconsistently, and the mechanisms are unclear. In the sheep infusion of oestradiol was associated with cervical softening and prostaglandin production. Inhibition of the oestradiol-induced prostaglandin production with meclofenamic acid (MFA) partially reduced the softening effect of oestrogen (Owiny, 1986). Other authors have concluded that oestradiol-17 β has a direct effect (not mediated by synthesis of PGs) on the physical and biochemical properties of the ovine cervix (Fitzpatrick and Dobson, 1981). In the rat, oestradiol can modify the mechanical properties of the cervix, but to a lesser extent than in physiological ripening, suggesting also an indirect effect (Maltier et al., 1993a). These experiments suggest the possibility of both a direct and an indirect action (through prostaglandin) of oestradiol in cervical softening. Oestradiol could upregulate prostaglandin production or the cervical sensitivity to prostaglandins. Stimulation of the production of relaxin by oestradiol has also been proposed.

Oestradiol had a positive effect on the production of prostaglandins by the rat uterus, but this effect has not been demonstrated in the ovine cervix (Ellwood et al., 1981). There is no direct evidence of upregulation of prostaglandin receptors by oestrogens either. This was suggested in women because the pharmacological potency of PGE $_2$ in the cervix increases with pregnancy status and during pregnancy (reviewed by Uldbjerg and Ulmsten, 1990). The cervix of postmenopausal women does

not respond to PGE₂ (Calder, pers. comm.) and PGE₂ binding sites were almost undetectable in a study carried out in postmenopausal women (Bauknecht et al., 1981). On the other hand, the concentration of PGE₂ binding sites in the uterus and the cervix did not change during the luteal or follicular phases of the cycle, and were higher in non-pregnant than in pregnant women (Giannopoulos et al., 1985; Adelantado et al., 1988). These results did not support the idea of upregulation of prostaglandin receptors by oestrogen. Alternatively, receptors may be less detectable at term because they are occupied by high levels of PGs stimulated by oestrogens, pretreatment with oestrogens results in a reduction of free PGE₂ binding sites in the myometrium (Bauknecht et al., 1981). In relation to a direct effect on the extracellular matrix of the cervix, both collagenase (guinea pig: Rajabi et al., 1990) and its inhibitor TIMP-1 may be stimulated by oestradiol-17 β (rabbit: Sato et al., 1991). Paradoxically, in sheep, oestrogens seem to inhibit the activity of collagenase (Elwood et al., 1981). In humans the clinical effect of oestradiol has also been equivocal when administered locally and intravenously on its own, or prior to PGE₂ (reviewed by Petersen et al., 1991).

1.6.2 PGF₂ α peak and other prostaglandins

In the sheep and other species, prostaglandins are thought to be the final mediators of parturition, causing cervical ripening and uterine contractions (Challis and Olson 1988). PGE₂ may be the initiator of labour in sheep, PG biosynthesis by the placental cotyledons increases after about 110 to 120 days of gestation (term: 147 days gestational age) (Thorburn and Rice, 1990; Thorburn, 1991). From about day 100 there is a progressive increase in PGE₂ concentrations in the maternal plasma (Thorburn and Rice, 1990; Thorburn, 1991). Although these early increases in prostaglandin synthesis coincide in time with the first microscopical and mechanical changes of the

cervix, the author is not aware of studies correlating the two.

There is a peak of $\text{PGF}_{2\alpha}$ metabolite (PGFM) in maternal plasma, 24 hours prior to labour, that follows in time the change in progesterone/oestrogen ratio, the maximal changes in compliance also occur on that day but probably precedes this peak (Fitzpatrick and Liggins, 1980; Owiny, 1986). Other prostagladins, including $\text{PGF}_{1\alpha}$ (PGI_2 metabolite), PGE_1 and PGE_2 remain at low level in the uterine vein blood (Liggins et al., 1977) and jugular venous plasma (Ellwood et al., 1981). But changes are observed in the cervical venous plasma, 6-oxo- $\text{PGF}_{1\alpha}$ starts to rise 24-36 hours before labour and peaks during labour and PGE_2 increases during 6 hours preceding delivery, supporting the idea of local production of PGE_2 and prostacyclin (PGI_2) in the cervix (Ellwood et al., 1981).

As in sheep, there is also a significant increase in circulating PGFM in women, the major metabolite of $\text{PGF}_{2\alpha}$, but an increase of PGE_2 metabolite has not been detectable (Mitchell et al., 1982; reviewed by Keirse, 1990). PGE_2 was expected to be high because it is ten times more potent than $\text{PGF}_{2\alpha}$ in the induction of uterine contractions and cervical ripening (Thiery, 1979). But a rise of both prostaglandins ($\text{PGF}_{2\alpha}$ and PGE_2) in amniotic fluid during labour and a direct correlation between these levels and cervical dilatation has been demonstrated (reviewed by Keirse, 1990; reviewed by Hayashi, 1993; reviewed by Maltier et al., 1993a). However, there has been some controversy as to whether $\text{PGF}_{2\alpha}$ and PGE_2 increase early enough in amniotic fluid to believe that they are causing cervical ripening and contractions, since a late rise may be a consequence rather than the cause of labour, this is a question of debate (McDonald and Casey, 1993; Romero et al., 1994).

Early studies of the prostacyclin metabolite $\text{PGF}_{1\alpha}$ were unable to show increased levels of this metabolite during labour, with improved

assays this has been shown in late pregnancy suggesting that increased production of prostacyclin may precede an increase in cervical ripening in women and sheep (Fitzpatrick and Liggins, 1980). Recent studies in sheep and women show an increasing capacity in the production of PGs from the last third of pregnancy with maximal levels observed immediately before labour, showing that the production of PGs drives labour rather than the other way around. The activity of PGHS-2, a rate limiting enzyme in the production of prostaglandins, increases in late pregnancy. This is an inducible enzyme, and in other tissues is known to be stimulated by proinflammatory mediators (Wimsatt et al., 1993; Texeira et al., 1993). There is also some evidence in sheep and women that the concentration of an endogenous inhibitor of PG synthesis (EIPS) decreases as term approaches (Porter, 1993). The synthesis of PGs in late pregnancy and labour is still a subject of discussion.

It is not clear whether peripheral levels of prostaglandins are difficult to measure or are not really controlling cervical ripening. However prostaglandins, with the exception of prostacyclin are known to be hormones of local control that are synthesised and released on demand (see next section on prostaglandin receptors). The idea of a paracrine control is supported by evidence of local production of prostaglandins by the ovine (Ellwood et al., 1981) and human cervix (reviewed by Hillier, 1990), an increase in the rate of production and a change in the pattern have been observed in association with parturition. Calder and Greer (1992) have suggested that while production within the cervix is important, the most potent sources of PGs may be the decidua ($\text{PGF}_{2\alpha}$) and the amniotic membrane (PGE_2), PGE_2 could reach the internal os of the cervix migrating across the chorion. Similarly, the ovine cotyledons are a well known source of PGs at late pregnancy (Porter, 1993).

Receptor studies in the cervix are confined to binding sites studies (reviewed by Rao, 1990) and the investigation of cAMP as a possible second messenger for PGE₂, both only in women (Norström et al., 1983). The PGE series seem to have greater affinity for its receptor than PGF₂α which may explain the higher potency of PGE₂ inducing contractions (Hofmann et al., 1983). Binding sites for prostaglandins of the E and F series have been found in the human cervixes. Binding sites for PGE₁ (Hofmann et al., 1983; Gianopoulos et al., 1985) and PGE₂ (Adelantado et al., 1988) were present in the cervix of premenopausal non-pregnant women undergoing hysterectomy due to various pathological conditions. PGF binding sites were reported to be present in the cervix during the cycle (Hofmann et al., 1983; Gianopoulos et al., 1985) but others found them during pregnancy and not during the cycle (Adelantado et al., 1988). The presence of PGE₂ and PGF₂α binding sites has been reported but they were almost undetectable in postmenopausal specimens (Bauknecht et al., 1981). Despite the important function of PGE₂ in cervical softening, in all these studies more binding sites were detected in the uterus than in the cervix. In the cervix more PGE₂ binding sites were observed in pregnancy (term) and the follicular phase than in the luteal phase; whereas in the uterus more binding sites were detected during the cycle (follicular phase > luteal) than at term (Adelantado et al., 1988). Binding sites results are difficult to interpret in relation to the function of PGE₂ in the cervix.

Further evidence for the physiological role of prostaglandins in cervical ripening is that inhibition of their synthesis can also inhibit cervical ripening at term or during labour in sheep (Mitchell and Flint, 1978; Owiny, 1986; Owiny et al., 1987), the effect occurring despite low levels of progesterone (Owiny and Fitzpatrick, 1992). PG synthesis inhibitors seem to have a similar effect in women (reviewed by Calder, 1990). PG synthetase inhibitors act on the synthesis (60%) but they may also act on

the receptors as antagonists since they bind to PGE₂ and PGF binding sites (Rao, 1990).

The pharmacological effects of prostaglandins in the cervix also support their physiological role. Prostaglandins have the capacity to ripen the cervix and to induce contractions in the uterus. There is experimental evidence suggesting that these two effects are independent. It is generally admitted that tocolysis with β -mimetics or physical disconnection of the two organs does not inhibit the cervical ripening effect of prostaglandins (reviewed by Challis and Olson, 1988; Owiny and Fitzpatrick, 1992; Calder, 1990). The effect of exogenous PGE₂ has been studied in microscopical, biochemical and mechanical studies during pregnancy (sheep: Fitzpatrick and Liggins, 1980; Stys et al., 1981; Owiny and Fitzpatrick, 1990; women: Conrad and Ueland, 1976; Uldbjerg et al., 1981; Uldbjerg et al., 1983; Rath et al., 1987; Osmer et al., 1991a; Greer et al., 1992; Rath et al., 1993). These studies have shown that PGE₂ induces changes in the cervix, similar to physiological ripening at term. An inflammatory response has been observed in the cervix following PGE₂ treatment (sheep: Fitzpatrick and Liggins, 1980; Owiny, 1986; women: Greer et al., 1992), with release of collagenase and elastase (Rath et al., 1993).

PGE₂ can induce cervical ripening in late pregnant sheep of 124-140 gestational age, the mechanical properties of the ovine cervix were changed with local application of PGE₂ and were comparable in magnitude with physiological ripening (Stys et al., 1981; Owiny and Fitzpatrick, 1990). In women, PGE₂ acts during the cycle, early pregnancy and late pregnancy, but sensitivity increases with gestational age (Uldbjerg and Ulmsten, 1990; Calder and Greer, 1992). In sheep both PGE₂ and PGF_{2 α} stimulate cervical ripening administered generally or locally with variable results (Fitzpatrick and Liggins, 1980). Direct comparisons of the two PGs have not been done in sheep, but in women they suggest that PGE₂ is more potent than PGF_{2 α}

(reviewed by Keirse, 1990). PGE₂ has also an effect relaxing cervical muscle *in vitro*, which is the opposite to the effect in the uterus (reviewed by Hillier, 1990).

The mechanism of action of PGF_{2α} and PGE₂ in the connective tissue of the cervix has not been completely clarified. Early work suggested that PGE₂ administration is not mediated by increased collagenase activity (Uldbjerg et al., 1983; Rath et al., 1987). Later, an increase in collagenase activity was found during a limited time period (only 1 and 2 hours) after the PGE₂ treatment (Osmers et al., 1991b). Changes in GAGs have also been reported (reviewed by Calder, 1994). *In vitro*, PGE₂ has been shown to stimulate pro-collagenase activity of the guinea pig cervix (Rajabi et al., 1990). Other authors have not been able to stimulate the collagenolytic activity of the human cervix *in vitro* and believe that cultures are almost devoid of PMLs and these cells may be the source of collagenase (reviewed by Uldbjerg, 1989). A current theory is that PGE₂ may act as a pro-inflammatory agent in synergism with IL-8, PGE₂ induces vasodilatation, and IL-8 attracts and degranulates the PMLs, releasing the proteolytic enzymes to degrade the matrix (Calder, 1994).

1.6.3 Oxytocin and its receptor (OTR)

Pharmacologically, oxytocin can induce contractions of smooth muscle of both the uterus and the cervix of the cyclic (Garcia Villar et al., 1984b) or the pregnant ewe (Owiny and Fitzpatrick, 1992). There is also some evidence that it may have an effect on the connective tissue of the cervix at term too. It reduces [³H] proline incorporation into uterine cervix proteins at term, suggesting inhibition of collagen synthesis. The effect on the connective tissue does not seem to be mediated by prostaglandin synthesis (reviewed by Hillier, 1990).

There is evidence of an increased release of oxytocin by the

neurohypophysis in late gestation with further release at labour in sheep. But in sheep and women oxytocin becomes detectable in peripheral blood rather late and after the onset of parturition, in the second stage of labour (at least several hours after the onset of labour contractions). There is a further increase with the passage of the fetus, cervical or vaginal distension stimulates hypothalamic release of oxytocin. The highest circulating levels are observed during the phase of fetal expulsion (reviewed by Challis and Olson, 1988; Maltier et al., 1993a; Porter, 1993). In most species the plasma increase is quite modest, considering its important role in inducing contractions during labour. Oxytocin receptors (OTR) are present in the myometrium and placenta, the number increase in late pregnancy with a surge just before labour and at the onset of labour. These results are consistent with early work on the increased sensitivity of the uterus to oxytocin at term in several species and together with the finding of an extra-hypothalamic source of oxytocin in the myometrium, led to the hypothesis that local regulation (production and increase in sensitivity) was more important than the levels of circulating oxytocin for the role of this hormone in the uterus (reviewed by Porter, 1993).

Peripheral changes of oxytocin occur too late to affect cervical ripening, but as in the uterus, local regulation may exist in the cervix too. Binding sites have been detected in the human cervix during the cycle (human: Fuchs et al., 1984 and 1985) and studied in non-pregnant sheep in detail (Matthews and Ayad, 1994). The receptor was present in the luminal epithelium, the superficial muscle layers and inner dense collagenous region, the presence of receptors was maximal at oestrus. The significance of this finding is unknown (Matthews and Ayad, 1994). But in the endometrium, Stewart et al. (1993) obtained partial codings of the ovine OTR cDNA, and they showed variations in the expression of the receptor and its mRNA during the cycle. The OTR and its mRNA is greatly

upregulated in the endometrium of non-pregnant sheep from day 12, which causes the release of the luteolytic PGF_{2α}. In pregnant sheep, trophoblast interferon is produced at the time of maternal recognition, which may inhibit the synthesis of endometrial OTRs protecting the *corpus luteum* from luteolysis (reviewed by Flint et al., 1992).

In cattle very low OTR concentrations have been detected in the cervix at mid-late gestation (175-250 days' gestation), but an increase was observed at 278 days of gestation (term 280-284) (Fuchs, 1994). These authors suggested that OTR may have a role in cervical ripening. OTR were found in the cervical mucosa, where they could mediate PGE₂ release (Fuchs, 1994), like endometrial OTR mediates PGF_{2α} release in women at term or in non-pregnant ruminant at the end of the cycle (reviewed by Porter, 1993). OTR mRNA increases were detected in the myometrium and endometrium of sheep at spontaneous or cortisol-induced labour (Wu and Nathanielsz, 1994). But OTR mRNA in the ovine cervix has not been detected using northern analysis at term (Wu and Nathanielsz, 1994). Similarly, the concentration of oxytocin receptors found in the human cervix during late pregnancy was very low and lower than in the uterus (Fuchs, 1994). There is evidence of the presence of OTR in epithelial cells, this was shown in scrapings of endocervical tissue from women at term (Kubota et al., 1994). It is possible that OTR are mainly sublocalised in the endocervix (Wu and Nathanielsz, 1994; Fuchs, 1994), the paucity of OTR in the muscular layer would prevent contractions and would facilitate relaxation during cervical ripening.

1.6.4 Relaxin

The physiological role of relaxin controlling cervical ripening in rats and pigs is not doubted (Sherwood et al., 1993). Because these two species are not very closely related phylogenetically, the action of relaxin in the cervix

may be a universal phenomenon in mammals, its role has been investigated in ruminants and women, but results are still inconclusive (reviewed by Sherwood, 1988 and 1994). The main source of relaxin in pigs and rats is the *corpus luteum*, the hormone is detectable in the peripheral circulation during the second half of pregnancy, and contributes to the growth of the cervix. Relaxin peaks in peripheral blood coinciding with the time of cervical ripening in pigs, rats and humans. In humans however, ovarian relaxin is not essential for cervical ripening and there is evidence of a paracrine action of relaxin produced in the placenta. Relaxin can also be produced in the uterus of rats and pigs (reviewed by Sherwood, 1988 and 1994).

Relaxin receptors have not been isolated in any species yet, but binding sites and second messenger responses have been demonstrated in the cervix of several species (pig, rat, human and guinea pigs), thus a paracrine regulation may be conserved between mammalian species (Sherwood, 1988; Dobson, 1988; Calder, 1990). There are morphological, mechanical and biochemical evidences to substantiate that relaxin is involved in cervical ripening, mainly in the pig and the rat but also in other species (Sherwood et al., 1993; Sherwood, 1994).

Surprisingly, comparison of the sequences of relaxin in a wide range of female vertebrates (pig, rat, human, monkey, shark, skate, whale, porpoise, horse, dog and guinea pig) has shown very low homology between some of them. Between mammals, for example, homologies of 30-60% have been observed and therefore ideally relaxin of the same species should be used when assessing the biological activity of this drug (Sherwood, 1994). Most studies however, have been done with purified porcine relaxin. There are differences between species in the major source of relaxin (*corpus luteum*, placenta or uterus) and secretory profiles. Apart from the relaxation of the cervix, other biological effects of relaxin includes:

inhibition of uterine contractions, relaxation of the pubic symphysis and promotion of the growth and development of the mammary apparatus. All these effects do not occur in all the species, but cervical ripening seem to be the most conserved among mammals.

There have been unsuccessful attempts to isolate and characterise ovine relaxin and its gene from the placenta (Wathes et al., 1988; Roche et al, 1993). A cDNA clone was isolated by RT-PCR using primers designed from areas conserved between pig, rat and human relaxin. But the clone was probably a pseudogene, because it contained many stop codons and mRNA expression was not demonstrable in ovary, placenta or endometrium (Roche et al, 1993). Studies using porcine relaxin (bioassays, RIA and immunoassays) have yielded confusing results and the main source of relaxin remains unknown in the sheep. Very low levels have been observed in plasma and tissues compared to other species with no increase throughout pregnancy (Renegar and Larkin, 1985; Wathes et al., 1988). Either poor cross-reactivity with porcine relaxin, extreme lability of the peptide or very low levels of gene expression in this species were suspected. However, recent studies have shown for the first time a modest peak of relaxin in peripheral plasma, occurring four day before normal parturition and using a RIA with porcine relaxin (Gazal et al., 1993). The situation in cattle is similar to the sheep, the bovine relaxin has been purified but not isolated from the ovary and remains uncharacterised. Heterologous RIA have shown only a small prepartum peak (reviewed by Taverne, 1992 and Sherwood, 1994). Pharmacological effects of porcine relaxin in the cervix have been also demonstrated in sheep and other species that will be reviewed later (section 1.8.3)

1.7 PHYSIOLOGICAL CERVICAL RIPENING AS AN INFLAMMATORY RESPONSE: INFLAMMATORY CELLS, CYTOKINES AND PROSTAGLANDIN E₂ (PGE₂).

Evidence is accumulating, mainly in humans but also in other species that, as proposed by Liggins more than a decade ago, cervical ripening in mammals consists of an inflammatory response (Liggins, 1981). Initially the involvement of inflammatory cells and prostaglandins was clear and in recent years the participation of cytokines has been incorporated into the theory.

1.7.1 Inflammatory cells

The theory that white cells have a role in the degradation of the extracellular matrix during cervical ripening in a general context of an inflammatory response was first proposed by Liggins more than a decade ago (Liggins, 1981). His theory was based on physical and chemical changes during cervical ripening in various species that resembled an inflammatory response. The emphasis in the early work, carried out in sheep and other species with light microscopy, was on the eosinophil, which was thought to have a great capacity to degrade collagen (Liggins, 1981). The finding of white cells, mainly PMLs, has been confirmed later in several species using TEM (sheep: Parry and Ellwood, 1981; guinea pig; Hegele-Hartung et al.: 1989). The leukocyte invasion in preterm sheep (at 140 gestational age) consisted of lymphocytes, monocytes and a few eosinophils, associating their presence with connective tissue breakdown (Fosang et al., 1984).

Polymorphonuclear Leukocytes (PMLs)

Human studies by Junqueira and colleagues showed extravascular PML surrounded by less dense collagen in what they judged to be “a halo of collagenolysis”, in those same studies evidence of collagen loss was demonstrated histochemically by staining collagen with picosirius red. A positive correlation was found between the number of infiltrating PML and the degree of collagenolysis (Junqueira et al., 1980). Biochemical findings of the presence of leukocyte elastase and its inhibitor antileukoprotease in the cervix changed with cervical ripening (Ito et al., 1980; Uldbjerg, 1989) confirming the theory that leukocytes are involved. Immunohistochemistry showed that the source of elastase during cervical ripening in humans is the PML (Kanayama and Terao, 1991).

Collagenase activity was later detected in degranulating PMLs during labour using an antibody specific for PML collagenase (MMP-8) that did not cross-react with fibroblast collagenase (MMP-1) (Osmers et al., 1992). This finding together with the inability to detect mRNA expression of the MMP-1 from fibroblasts, led to the following suggestion by Osmers and colleagues: the cell that secretes “activated collagenase” found in the cervix during labour is the PML rather than the resident fibroblast. Using activity-based assays, the two collagenases are indistinguishable and others think that the matter has not been clarified. The collagenase activity detected in the cervix, could be MMP-8 or MMP-3, MMP-3 activates the collagenase of the fibroblast (MMP-1) (Leppert, 1992). Recent work using a very sensitive ELISA with monoclonal antibodies specific for MMP-1 failed to detect increased activity in relation to cervical ripening, and the authors suggest that the increased collagenase activity found by others with activity-based assays is probably attributable to MMP-8 (Morrison et al., 1994).

Several inflammatory mediators, known as the classical “chemoattractants” (named for their ability to stimulate chemotaxis) can

activate the PML acting on specific receptors, they include bacterial products like N-formyl-methionyl-leucyl-phenylalanine (fMLP), cytokines like interleukin 8 (IL-8), products of the complement cascade (C5a) and lipid mediators like platelet activating factor (PAF) and leukotriene B₄ (LTB₄). Upon recognition of any of these agents, chemotaxis, secretion of proteolytic enzymes and production of reactive oxygen intermediates (ROI) occurs (Baggiolini et al., 1992, Baggiolini et al., 1993; Doherty and Janusz, 1994). ROI released by activated PMLs promote degradation of the extracellular matrix by inactivating antiproteases (see previous leukocyte elastase section) and by autoactivation of metalloproteinases (MMP-8 and MMP-9). The PML collagenase (MMP-8), unlike MMP-1 does not require cleavage by MMP-3 for activation, but the release of hypochlorous acid during the respiratory burst as part of the effector responses of PMLs, a similar mechanism exists for gelatinase (MMP-9) (reviewed by Woessner, 1991; Leff and Repine, 1993; Doherty and Janusz, 1994). The intracervical administration of IL-8 induces cervical ripening (Chwalisz et al., 1994; El Maradny et al., 1994), suggesting that the activation of PMLs leads to cervical ripening. Summarising, there is evidence to support the role of two PML enzymes in cervical ripening, leukocyte elastase (Kanayama and Terao, 1991) and collagenase (MMP-8) (Osmers et al., 1992).

Peripheral changes in white cells counts have been observed in sheep and cattle during pregnancy and parturition (Anosa and Ogbogu, 1979; Kehrli et al., 1989). In humans there is evidence of PML activation, as measured by superoxide production, which is increased in peripheral blood of pregnant women compared to non-pregnant (Okamura et al., 1988). Activated PMLs did not increase with the advancement of pregnancy in blood but did increase in the cervix (Okamura et al., 1988).

Eosinophils

We have already mentioned that the number of eosinophils counted per high power field increased greatly in ovine cervical preparations (Fosang et al., 1984), similarly a ten fold increase was observed in guinea pigs (Liggins, 1981). Activated and degranulated eosinophils have been observed in cervical biopsies of pregnant women at term (Knudsen et al., 1991) and in rats (Luque and Montes, 1989) surrounded by halos of collagenolysis. The contents of the eosinophilic granules have been studied in women (reviewed by Venge, 1993), but it is not clear whether cationic proteins or proteinases are released during the degranulation observed microscopically during cervical ripening (Luque and Montes, 1989; Knudsen et al., 1991). The release of eosinophilic major basic protein has been detected by immunohistochemistry in rat cervixes (Duchesne and Badia, 1992).

Eosinophils are also known to contain several proteinases including an interstitial collagenase capable of degrading the major collagen types of the cervix, leukocyte elastase and gelatinase B (MMP-9) (Hibbs et al., 1982; Lungarella et al., 1992; Stähle-Backdahl and Pärks, 1993). Unlike the PML, the eosinophil not only stores proteinases, but may have the capacity of synthesising them, as in the case of MMP-9 in disease states (Stähle-Backdahl and Pärks, 1993). It has been proposed that the eosinophil may degrade collagen in conditions like granulomas, fibrosing alveolitis, chronic inflammatory disorders of the lung and metastasis (Luque and Montes, 1989; Stähle-Backdahl and Pärks, 1993). But the activity of these enzymes has not been studied in relation to cervical ripening or in the pathogenesis of other diseases implicating the eosinophil (asthma), which have put more emphasis on the cationic proteins (Venge, 1993; Butterfield and Leiferman, 1993).

Infiltration of eosinophils is regulated by steroids in the rat cervix (Luque and Montes, 1989). Eosinophilia and eosinophil activation are regulated in inflammatory responses by chemokines of the CC type mainly (RANTES, Eotaxin, MCP-2, -3 and MIP-1 α), IL-5, the lipid mediators (PAF, LTB₄ and prostaglandins) (Giembycz and Barnes, 1993). The importance of these mediators in the activation of eosinophils leading to cervical ripening has not been evaluated yet.

Mast cells

Mast cells are resident cells of the connective tissue, strategically located near the surfaces exposed to the external environment. They are replete of mediators of the inflammatory response and designed to release some of them rapidly. It is the only resident cell type capable of storing tumour necrosis factor (TFN) in cytoplasmic granules that is rapidly released (within 10 to 20 min of challenge). Some authors therefore see them as the initiators of the inflammatory response both in natural immunity (i.e. against bacteria) or acquired immunity (parasites and allergic diseases). The mast cells can respond rapidly because unlike the PML or the macrophage respectively, it does not require recruitment or synthesis of TFN (reviewed by Galli, 1993).

The granular contents of the human mast cells include a high content of a proteoglycan, heparin, responsible for the metachromatic staining of these cells. The GAGs of heparin are combined with neutral proteases (tryptase, chymase or carboxypeptidase) forming a crystalline structure in the granules. Tryptase is the major protein of mast cells in humans, whereas in the murine cells, chymases predominate (Caughey, 1994). Heparin is also combined with the basic amine histamine. Histamine, heparin and neutral proteases are the major mediators stored by the mast cell. Other substances stored in smaller quantities include:

exoglycosidases, chemotactic factors (eosinophil and PML), oxidative enzymes and kininogenase. These cells have also an action in the degradation of the extracellular matrix releasing tryptase and chymase which activate other lytic enzymes (like procollagenase and prostromelysin) (reviewed by Knudsen et al., 1991). They are also involved in the degranulation of the eosinophil (reviewed by Knudsen et al., 1991).

Mast cells have been noted in the ovine (Aughey, personal communication), guinea pig (Hegele-Hartung et al., 1989), and human cervical tissues at term (Lo Stumbo and Pellegrini, 1970). It has been proposed that the mast cells could participate in cervical ripening by attracting and activating cells that contain collagenase and other proteinases (PML and eosinophil) (Knudsen et al., 1991) or with the release of vasoactive substances (Lo Stumbo and Pellegrini, 1970). The presence of these cells in the uterus seem to change under the influence of oestrogens and androgens (reviewed by Knudsen et al., 1991).

Monocytes/Macrophages

These are resident cells of the connective tissue that participate in acute and chronic inflammatory responses and host defence against both bacteria and neoplastic cells, releasing proinflammatory mediators and proteinases (reviewed by Gallin et al., 1992). The macrophage secretes an extensive repertoire of products, most of them are newly synthesised and inducible (collagenase, elastase, plasminogen activator, $\text{TNF}\alpha$, LTC_4 , PGE_2 , $\text{PGF}_{1\alpha}$, PGI_2 , PAF, hydrogen peroxide, nitric oxide, superoxide anion, etc.), but some are synthesised constitutively or are preformed and stored in granules (reviewed by Gallin et al., 1992). This cell is an important source of PGE_2 in inflammatory responses. In acute inflammation, it is also

capable of clearing the inflammatory site of debris by phagocytosing apoptotic PMLs (Savill and Haslett, 1993) and eosinophils (reviewed by Gallin et al., 1992).

Macrophages have also been reported in the guinea pig during spontaneous cervical ripening (Hegele-Hartung et al., 1989) and in the human cervix (Lo Stumbo and Pellegrini, 1970; Rayburn et al., 1994). In the sheep macrophages and lymphocytes were more prominent than eosinophils in late pregnancy (after day 140 and before term) simultaneous with the first evidence of tissue breakdown; this ratio changed at term (Fosang et al., 1984). Unlike the PML, there are no demonstrations of the monocyte/macrophage enzymatic activity during cervical ripening, although it is known that they release proteolytic enzymes in other tissues.

Serine proteinases may be lost during maturation from monocytes to macrophages, the elastolytic activity in monocytes is due to a serine proteinase and in macrophages to a MMP (Campbell et al., 1991c). Monocytes store serine proteinases in granules, less than PMLs but still considerable amounts, which may be useful for migration or rapid responses (reviewed by Caughey, 1994; Campbell et al., 1989; Kargi et al., 1990; Campbell et al., 1991c). In arthritis, macrophages are thought to contribute directly with release of MMPs and also by producing inflammatory mediators that regulate MMP production by the fibroblast. The secretion of collagenase and macrophage elastase (MMP-12) is inducible in macrophages, taking 24 to 48 hours (reviewed by Henson et al., 1992). Unlike the PML, macrophages do not store collagenase (Birkedal-Hansen et al., 1993). Macrophages, like other leukocytes have also receptors for oestrogen and progesterone (reviewed by Gallin et al., 1992), this aspect has not been studied in relation to cervical ripening.

Lymphocytes

Some authors have reported increased number of lymphocytes, associated with macrophages and a few eosinophils (Fosang et al., 1984), but others do not report a substantial change at the time of cervical ripening. They secrete mainly gelatinases (MMP-2 and 9) in small amounts that may allow migration through basement membranes. They are not sources of elastase, but other serine proteinases like granzyme A, B and met-ase (reviewed by Caughey, 1994).

Fibroblasts

Fibroblasts like white cells, can degrade the matrix. The repertoire of MMPs is similar to the macrophage (MMP-1, -2 and stromelysin) but more limited (no MMP-9 is produced), and the levels of production are higher in the fibroblast (reviewed by Gallin et al., 1992). We include the fibroblast in this section too because the release of collagenolytic and elastolytic activities, due to MMP-1 and MMP-2 respectively, can be induced from these cells in the skin by inflammatory mediators (Okada et al., 1993). PGE₂ is a potent inducer of fibroblast collagenase (MMP-1) mRNA levels, promoter activity and secretion in the skin (Mauviel et al., 1994).

1.7.2 Cytokines

As to how inflammatory cells are recruited into the ripening cervix at term and activated, there is growing evidence for the involvement of cytokines in man, the rabbit and the guinea pig. Cytokines are well characterised in mouse and man, but in sheep this work has only started in the 1990s (reviewed by McInnes, 1993). Cytokines are proteins participating in cross-talk between white cells, endothelial cells and other cells of the body in inflammatory reactions (reviewed by McInnes, 1993).

Cervical explants from pregnant rabbits and pregnant women

produce great amounts of the cytokines interleukin 1 (IL-1) and interleukin 8 (IL-8) (Ito et al., 1987, 1988; Uchiyama et al., 1992; Barclay et al., 1993). IL-8 is a secondary mediator of inflammation, acting at later stages of the inflammatory process. IL-8 causes the PML to degranulate, releasing MMP-9 from secretory vesicles, MMP-8 from specific granules and elastase from the azurophil granules (Baggiolini et al., 1989). The release of these enzymes has been implicated in cervical ripening (see previous sections). The mRNA expression of IL-8 can be upregulated by early mediators like IL-1 and tumour necrosis factor α (TNF α).

For the above reasons the involvement of TNF α , IL-1 and IL-8 in cervical ripening has been studied in several species, finding that they can induce cervical ripening (rabbit: El Maradny et al., 1994; guinea pig: Chwalisz et al., 1994). These findings further support the physiological role of these cytokines and of the PML in cervical ripening (see section 1.8.5, later for more details on these treatments).

There is some information on the regulation of interleukins and the degradation of the extracellular matrix by white cells. Progesterone is an anti-inflammatory hormone that may control cytokine production in various intrauterine tissues, including the cervix (Hegele-Hartung et al., 1989; Ito et al., 1994, see other section on progesterone withdrawal, page 35). Other authors see the fibroblast as the main cell in cervical ripening and suggest that hyaluronic acid which increases in late pregnancy could stimulate the production of IL-1, which can in turn stimulate the production of collagenase by fibroblasts (reviewed by Leppert, 1992).

1.7.3 PGE₂ as a lipid mediator of the inflammatory response

Lipid mediators, derived from cell membranes, such as the platelet activating factor (PAF) and eicosanoids (prostaglandins and leukotrienes) modulate locally the inflammatory responses (Gallin et al., 1992). Liggins proposed that PGE₂ acts in the cervix as a proinflammatory mediator, like in any inflammatory response, probably producing vasodilatation, which was the most accepted mechanism at the time (Liggins, 1981). The mechanisms of action of PGE₂ in the inflammatory response, however are still not clear, because exogenous PGE₂ can exert both proinflammatory and anti-inflammatory actions (Phipps et al., 1991; Weissman, 1993). Some authors are trying to explain the pro and anti-inflammatory effects in terms of different prostaglandin receptor subtypes that mediate one or the other (Coleman et al., 1990; Wise and Jones, 1994). Unfortunately studies on the mechanisms of action of the anti-inflammatory actions of PGE₂ are better documented than the proinflammatory actions (Reibman et al., 1990; Gryglewski and Salvemini, 1992).

A synergism has been demonstrated between IL8 and PGE₂ to induce PML influx in the rabbit skin. This proinflammatory action of PGE₂ is thought to be due to its high potency as a vasodilator and a similar mechanism has been proposed in the cervix (Kelly, 1994). PGE₂ may induce plasma exudation by reducing the tone of the smooth muscle of the blood vessels. Since EP₂ and EP₄ mediate relaxation of the smooth muscle this action may be mediated by these receptor subtypes.

A new finding is that membrane receptors for PGE₂ are not only localised in blood vessels, but also in white cells, which can also release PGE₂ (Gallin et al., 1992; Coleman et al., 1990). PGE₂ could also cause vasodilatation by acting directly on PMLs (Armstrong, 1992) which have the capacity of inducing exudation (Armstrong et al., 1990; Wedmore and Williams, 1981). PGE₂ on its own also has chemoattractive properties for

PMLs and other white cells in a variable fashion depending on the species. It is possible that similar synergism exists between PGE₂ and monocyte chemotactic factor (MCP-1) in the recruitment of monocytes (reviewed by Kelly, 1994). PGE₂ has also been implicated in the production of matrix metalloproteinases by monocytes/macrophages (Corcoran et al., 1992) and fibroblasts (Mauviel et al., 1994).

1.8 CLINICAL INDUCTION OF CERVICAL RIPENING

In this section the compounds and methods that induce ripening are listed to give an overview of how to approach the cervical ripening problem. In ruminants, very little has been published, but recent work is addressing a pharmacological approach for cervical ripening:

- (1) transcervical artificial insemination (in sheep),
- (2) ringwomb which apart from the sheep, can affect buffalo, cattle and goats (reviewed by Dobson, 1988; Ghosh et al., 1992) and
- (3) transcervical embryo transfer (in heifers, goats and sheep).

The subject is documented very extensively in human obstetrics for termination of pregnancy in the first two trimesters and induction of labour in cases of “unfavourable” cervixes (reviewed recently by Hayashi, 1993). This information can be applied to other species. However, the dilatation of the cervix during the cycle, the most relevant topic for this thesis, has not been extensively addressed in women.

In laboratory animals, work has been done trying to clarify the mechanism of action of the different methods used in human obstetrics (rat: Saito et al., 1981; guinea pig: Chwalisz et al., 1991). Much work has been done on relaxin in guinea pigs, rats, and pigs, since its action on the cervix in those species is better known than in other species, including the humans. Comparison between methods have been done in rats (Saito et al., 1981) and women (Gupta and Johnson, 1992), but not in domestic species.

1.8.1 Prostaglandins and prostaglandin analogues

Prostaglandins of the F series for intramuscular (i.m.) administration are the only prostaglandins that are commercially available for veterinary use in reproduction. This type of preparation induces luteolysis and has found many applications in reproduction (Arthur et al., 1989). Intramuscular

preparations of PGF_{2α} (Prosolvin, Intervet) have been tested experimentally to treat cases of ringwomb in goats with considerable success (80%), 7.5 mg were administered (Majeed and Taha, 1989 a & b). The PGF_{2α} analogue Cloprostenol (Estrumate, Coopers) produced satisfactory results (78%), using 500 mg (Ghosh et al., 1992). But because of the route used and because this PG is luteolytic in pregnant goats, it is not possible to know if the treatment had worked directly on the cervix (Fitzpatrick and Liggins, 1980) or by inducing luteolysis and progesterone withdrawal (Arthur et al., 1989).

Early experimental work used both PGE₂ and PGF_{2α} to induce cervical ripening in late pregnant sheep (day 125, i.e. about three weeks before term), as an experimental model for obstetricians. These prostaglandins were first infused into the posterior aorta but because the effect was variable and in general antagonised by high peripheral progesterone levels, the intracervical route was tested, being more effective (Fitzpatrick, 1977). Human clinical studies have also shown that local preparations are more suitable to induce cervical ripening and avoid side effects (Calder, 1994), but there are no suitable commercial preparations of this type for veterinary use. In human obstetrics, both PGF and PGE series (PGE₁ and PGE₂) were initially found to be effective in the induction of cervical ripening, but lower doses of PGE were effective and therefore the use of the E series dominates. The E series is not known in veterinary practice, but human local preparations of PGE₂, have been recently tested in small ruminants for cervical ripening in order to develop transcervical techniques of ET and AI. In cattle, these techniques can also be improved with PGE₂ in heifers with small cervixes (Humblot, personal communication).

1.8.1 I Prostaglandin E₂ (PGE₂) used in humans

Local application (intracervical or vaginal) of PGE₂ is the better known and most widely used method to induce cervical ripening in human obstetrics (reviewed by Keirse, 1990). The therapeutic action is believed to be related to their prominent role in physiological ripening. One problem of prostaglandins is their ubiquitous actions, leading to side effects. This problem has partially been resolved by using a local route of administration, that allows reduction of the dose needed, and the development of prostaglandin analogues (reviewed recently by Coleman et al., 1990; Calder, 1994). Another problem is their short half life, but analogues of a longer half life than the natural compounds have been developed and are therefore more potent, being indicated for early stage pregnancy termination or for exploratory purposes during the cycle (reviewed by Bygdeman, 1992; Calder and Greer, 1992). The clinical use of prostaglandins and their analogues will be expanded in the next section (1.9).

There are several preparations for human use in Obstetrics (Monthly Index of Medical Specialities; Martindale, 1993). The natural compound, PGE₂ (Dinoprostone) carried in tablets, lipid-based suppositories, embedded in pessaries and mixed in gels has been applied intravaginally (Prostin E₂, Upjohn) in dosages ranging from 1-5 mg for ripening the cervix prior to induction of labour. PGE₂ can also come in a viscous gel, containing 500 micrograms in prefilled syringes for intracervical use (Prepidil, Upjohn). Sulprostone is a synthetic analogue of dinoprostone (PGE₂) for i. m. use. Carbaprost is synthetic analogue of dinoprost (PGF₂α) for intravenous and i. m. use. Gemeprost and Misoprotol are synthetic analogues of alprostadiol (PGE₁), they are used intravaginally and orally respectively. Misoprotol is an oral preparation because it was originally manufactured for the treatment of duodenal and gastric ulcers, but it was

later found to be a cheap and accessible drug (for those countries where termination of pregnancy is forbidden) for the induction of cervical ripening. Gemeprost and Sulprostone are well known but the dose of Misoprostol is still being investigated (reviewed by Bygdeman, 1992; Calder and Greer, 1992;).

1.8.1 ii Prostaglandin E₂ (PGE₂) used in sheep

It has been known for some time that prostaglandins of both the E and the F series, have a physiological role in cervical ripening and also have a demonstrated pharmacological effect at term (Stys et al., 1980; Fitzpatrick and Liggins, 1980; Stys et al., 1981; Ledger et al., 1985; Owiny and Fitzpatrick, 1990; Owiny and Fitzpatrick, 1992). This is further reviewed in a following chapter (chapter III).

During the cycle, the effect of PGE₂ in the ovine cervix is now being investigated. Rickords and White (1988) used a lipid-base suppository for intravaginal human use, containing 10 mg of PGE₂ (Dinoprostone), and showed that it can induce partial ripening in anoestrous sheep, mostly at the external os in most cases. Complete dilatation and catheterization was possible only in one ewe out of 20 animals. South African workers have reported very successful transcervical embryo collection in anaesthetised virgin and adult sheep (Barry et al., 1990) and goats (van Niekerk et al., 1990). On day 6 following oestrus, oestradiol cypionate (1 mg) was administered intramuscularly in conjunction with 1 mg of PGE₂ (Prostin E₂, Upjohn), dissolved in saline and given intracervically. Using a similar method, results were not as successful in Scotland (Mylne et al., 1992). Parity and the breed affected the success in passing the cervix, ease of penetration was better in Texels than in Suffolks (53%-28% respectively) and very poor in virgin sheep (6%). If cervical penetration was successful, the collection of embryos was comparable to laparoscopic collection (60%-

58% respectively).

The prostaglandin analogue Misoprostol, administered intravaginally has been used to facilitate transcervical artificial insemination with frozen semen in Brazil (Mies Filho et al., 1993). The use of this drug may appeal to veterinary use because is very cheap, the human dose (200 µg to 1 mg cost about £4) and no refrigeration is necessary. It is an oral preparation, but in this work 100 µg of the PGE₂ were administered intravaginally dissolved in saline, as a liquid solution or impregnated in sponges. Preliminary results with the aqueous solution revealed total success in catheterising the cervical canal 36 hours after administration. But with the sponges, only partial catheterization was possible in 15% of the animals (n=48) and the uterus was not reached in any animal (Mies Filho et al., 1993).

1.8.1 iii Prostaglandins used in heifers, cows and mares

The effect of prostaglandin E₂ has also been studied in non pregnant (day 8-10) and early pregnant (33-40 days gestation) heifers. A gel for human use (Minprostin, Upjohn, Sweden), containing 2 mg or 6 mg, was administered intracervically to non-pregnant and 2 mg to the pregnant heifers. There was dilatation of the cervix from 3 hour after treatment, as assessed by means of Hegar dilators of increasing diameters. The 2 mg dose appeared to be sufficient. There was systemic absorption that was more rapid than in women, but the effect was purely local. The viability of the embryo was not affected by the treatment, which was monitored by ultrasonography (Duchens et al, 1993).

Others have used higher doses of PGE₂, 20 mg of intracervical Dinorprostone (Prostin, Upjohn), in cows to flush live fetuses in early pregnancy (33-42 of pregnancy) with variable success; the cervix was very dilated (14 cm) but fetuses died in some cases (Lavoir and Betteridge,

1992). Both PGE₂ and PGF_{2α} have also been used in cows (Zerobin et al., 1973) and mares (Volkmann and De Cramer, 1991) to terminate pregnancy.

1.8.2 Progesterone antagonists and steroids

1.8.2 i Progesterone antagonists

Progesterone antagonists (antigestagens) act by competing for receptors with progesterone, either by having more affinity for the receptors or by being present in greater concentrations. They also present high affinity for glucocorticoid and mineralocorticoid receptors, causing undesired side effects. Mifepristone, also known as RU 486 (Mifegyne, Roussel) was the first antigestagen to be developed, followed by its analogues ZK 98.299 (Onapristone, Schering) and ZK 98.734 (Lilopristone, Schering) of lower antiglucocorticoid activity (Chwalisz et al., 1991).

Progesterone withdrawal is an important stimulator of cervical ripening (see section 1.6.1i, page 33) and progesterone antagonists are able to induce ripening in various mammalian species (sheep, rats, rabbits, pig, cow, guinea pig, lower primates and humans) (reviewed by Chwalisz, 1994). Onapristone has shown ripening activity in the guinea pig and Lilopristone was very effective in the rat, this effect can be achieved without the stimulation of uterine contractions (Chwalisz et al., 1991). These are novel drugs in human obstetrics, a ripening effect of oral Mifepristone has been reported in pregnant and non pregnant women (Reviewed by Chwalisz, 1994).

The use outside pregnancy may be interesting for AI and ET in domestic animals. Although RU 486 has been tested in cows and sheep, the purpose of these trials was not the induction of cervical ripening only, but the induction of full labour, both cervical ripening and myometrial

contractions. RU 486 decreased circulating progesterone concentrations within 24 hours in late pregnant sheep (Gazal et al., 1993) and cows (Li et al, 1991) and induced premature safe labour in sheep (Gazal et al., 1993).

1.8.2 ii Oestrogen

Oestrogen promotes cervical ripening in sheep and other species (Stys et al., 1980; Owiny, 1986; Steiner and Creasy, 1983; Robinson et al., 1991). The local application of oestradiol, is not the method of choice in women, but it is indicated in the presence of unexplained bleeding, when prostaglandins are not recommended (reviewed by Hayashi, 1993). Other authors believe that neither the effect nor the mechanism of action of oestrogens have been well studied in women yet (reviewed by Petersen et al., 1991). In sheep it has been used to prime PGE₂ and oxytocin treatments (Barry et al., 1990; Khalifa et al., 1992).

1.8.2 iii DHEAS (dehydroepiandrosterone sulphate)

The rationale of using this androgen is that physiological concentrations of DHAS may contribute to cervical ripening in women, increases have been observed in the cervix with gestational age. Levels are also higher in women with ripe cervixes, compared to unripe cervixes (reviewed by Hillier, 1990). Pharmacologically it has been administered intravenously effectively in women. The administration induced an increase in collagenase and β oestradiol (reviewed by Hayashi, 1993). We are not aware of the involvement of DHEAS in physiological ripening of other species.

1.8.3 Relaxin

Relaxin has a demonstrated physiological role in the dilatation of the cervix and separation of the pubic symphysis in some species (rats and pigs: reviewed by Sherwood, 1994). But, the physiological role of relaxin during parturition has not been clarified in species like sheep, cattle and humans (see section 1.6.4, page 46).

Since the 1930s relaxin was extracted from pregnant sows' ovaries and used experimentally in other species, these extracts were not fully purified until the late 1970s. The use of relaxin to induce ripening is a method under investigation at the present time in human obstetrics. Porcine relaxin administered by intravaginal or intracervical gel has been found to be useful to induce cervical ripening in women at term without increasing the uterine contractility (reviewed by MacLennan et al., 1991). But paradoxically, recombinant human relaxin (Genentech Inc. USA), which has recently become available, had no appreciable effect on cervical ripening in women (Brennand et al., 1993). These preliminary results were obtained in Scotland, using an intravaginal gel (containing either 0,1,2, or 4 mg of relaxin).

In ruminants only porcine relaxin has been used (pRLX) for the induction of cervical ripening. In cattle, purified porcine relaxin (3000 U/mg) administered either into the cervical os or intramuscularly, seem to have a pharmacological action ripening the cervix at term (Perezgrovas and Anderson, 1982; Bagna et al., 1991). An extract of relaxin was also effective 5 days post oestrus in cows primed with oestrogen, several doses were titrated (Graham and Dracy, 1953). This type of regime produced histological changes similar to those seen at labour (Eggee and Dracy, 1966).

In sheep, the action of porcine relaxin is not clear, similar or higher doses than in cattle do not always produced a positive result, 0.5 mg pRLX (>3000 U/mg) intramuscular following oestrogen priming with or without progesterone, increased the efficacy of transcervical catheterisation in ovariectomized ewes (Nemec et al., 1988). Akinbami and colleagues (1990) found that relaxin (3000-4500 U per ewe) was totally effective or ineffective facilitating cervical penetration, depending on the postpartum stage of the ewe.

Salamon and Lightfoot (1970), using doses of 100 to 12500 U per ewe, did not achieve deeper insemination, when relaxin was given 1 and 12 hours after the onset of oestrus.

1.8.4 Oxytocin

It has been suggested that oxytocin may change the connective tissue of the cervix (Hillier, 1990), but this action has not been exploited clinically in human obstetrics and oxytocin is mainly used to induce uterine contractions, when the cervix is already ripened physiologically or pharmacologically by other drugs. Receptor studies have suggested a physiological role of oxytocin in cervical ripening of cattle, but this type of evidence has not been found in sheep (see previous section: 1.6.3, page 44). But, exogenous oxytocin given intravenously dilates the cervix in sheep for artificial insemination and for ET (Khalifa et al., 1992). The use of this drug was based on empirical observations of this effect and because the existence of receptors for oxytocin was predicted at progestogen-induced oestrus and following the administration of oestradiol on day 9. Studies in Canada using oxytocin for transcervical artificial insemination with frozen semen have also shown very good results in penetration of the cervix (90%), but poor fertility (Castonguay, personal communication).

1.8.5 Cytokines: recombinant human IL-8, IL-1- β and TNF- α

Recombinant human cytokines have not been used clinically, but their apparent physiological role and the results of local application in laboratory animals suggests that they may become a treatment in the future, specially IL-8 (Calder, personal communication). One advantage of this treatment over PGs is that they do not induce myometrial contractions (El Maradny et al., 1994).

Recombinant human interleukin-8 (Oncogene Science) when administered vaginally (suppositories of 100 ng for 5 days) can induce cervical ripening in nonpregnant and pregnant rabbits (El Maradny et al., 1994). Similarly, Chwalisz and colleagues (1994) purified 3 human recombinant interleukins (IL-8, IL-1 β and TFN- α) and confirmed the appropriate specific activity of each of them. Local application (intracervically) of these interleukins produced cervical ripening in advanced pregnant guinea pigs, the IL-8 and IL-1 β effect being more similar to physiological ripening.

1.8.6 Mechanical methods

For decades cervical widening by mechanical means has been known to be effective. The mechanism is not fully understood but some clinicians have successfully used mechanical devices to dilate the unripe cervix alone or combined with pharmacological methods in women (Steiner and Creasy, 1983). Similarly, in veterinary practice (sheep, goats and cattle) a finger or the hand of the operator has been the most common method to solve a dystocia due to failure of the cervix to dilate (Arthur et al., 1989).

Metal cylinders

The introduction of metal cylinders of increasing diameter is a traumatic method that had been used in the past in women (reviewed by Fisher et al., 1981) and is still used in cattle for ET (Cattoni, personal communication).

Intracervical tents

This is an old fashion method that is still used and is very acceptable in obstetrics (reviewed by Gupta and Johnson, 1992; Hayashi, 1993), it was first described in the literature in the middle of the nineteen century in Glasgow Med J (Sloan, 1863). Intracervical tents are hydrophilic cylinders (natural or synthetic) to be introduced into the cervical canal. They work partially by attracting water, the tent swells 2-4 times its original size and causes the cervix to dilate very effectively, but with considerable discomfort.

Natural tents are kelp (seaweed) stems, measuring 2-6 mm in diameter and 60 mm in length, that are cut dried and sterilised. The first use of these method describes the use of *Laminaria digitata* (Sloan, 1863). *Laminaria japonica*, has also been used (Hayashi, 1993). Synthetic materials have been developed that act in the same way but faster: Lamicel and Dialapan, both registered trade marks, are more hygienic and also permit better control of the swelling, they do not elongate unlike the natural material and therefore there is not risk of entrapment of cervical tissue or neighbouring structures.

Lamicel is a polyvinyl alcohol sponge, impregnated with magnesium sulphate and compressed to a long thin cylindrical tent (available in two sizes: 3 or 5 mm thick and 75 mm long). When the tent absorbs water it swells to four times its original size. Dialapan is made of hypan (polymerised, polyacrylnitrate hydrogel). It comes in 3 sizes (4 x 55 mm, 4 x 65 mm and 3 x 55 mm). It seem to be the fastest and most potent.

Intracervical tents are effective cervical dilators in women in therapeutic abortion (in the first and second trimester) and before the induction of labour. Lamicel dilates the cervix at term within 3-4 hours with a maximum effect within 6 hours. The mechanism of action of these tents has not been fully understood. Apart from the possible passive stretching of the cervix, active dilatation seems to be induced by the tent, since the diameter of the cervix is greater than the tent after the treatment. Various mechanisms so far investigated have not been proven: the tents do not seem to exert significant radial pressure, increase plasma prostaglandins or cause inflammation. Mechanisms of action by magnesium sulphate, relaxing smooth muscle or influencing cervical water balance have also been disputed. There are reports of collagen separation observed in electron microscopy induced by Lamicel and Dilapan (Norström et al., 1988). We are not aware of the use of this method in livestock or companion animals.

Balloon catheter with extra-amniotic saline infusion (BCEAS)

A catheter with a balloon attached to the tip, is inserted into the cervical canal until the balloon is located at the internal os. The balloon is then distended with water to dilate the canal. The method is effective but is not recommended at term because of the risk of infection in women (reviewed by Hayashi, 1993).

1.9 MOLECULAR STUDIES OF THE PROSTAGLANDIN E₂ (PGE₂) RECEPTOR (EP₃) SUBTYPE EP₃

1.9.1 Nomenclature and classification of prostaglandins and their receptors

Prostaglandins (PGs) and several related substances, like thromboxane A₂ (TxA₂), prostacyclin (PGI₂), hydroxyeicosatetranoic acids (HETEs) and leukotrienes (LTs), constitute a family of unsaturated fatty acids, all with a 20-carbon skeleton, collectively known as eicosanoids (from the Greek word eicosa, meaning 20). Eicosanoids and platelet activating factor (PAF) modulate inflammatory responses and, in those contexts, they are also known by the collective name of lipid mediators, because they all derive from the membrane phospholipids. Biologically active eicosanoids are formed by two major pathways, namely the cyclo-oxygenase and the lipoxygenase; in addition a third pathway designated epoxygenase has more recently been discovered. PGs, thromboxanes and PGI₂ are formed by the cyclo-oxygenase pathway, while the lipoxygenase products include HETEs and leukotrienes (so named because of their formation in leukocytes) (reviewed by Mitchell, 1990; Urich, 1990).

Prostanoids is a term that collectively include the products of the cyclo-oxygenase pathway, they are produced in three steps: 1) release of fatty acid (primarily arachidonic acid), 2) conversion of arachidonate to a common precursor (PGH₂) and 3) isomerization of the individual prostanoids. In the first step, arachidonic acid is released from the membrane phospholipids by the action of phospholipases. Arachidonic acid is then converted to an unstable endoperoxide intermediate (PGH₂) by the cyclooxygenase prostaglandin H synthase, either a constitutive (PGHS-1) or an inducible (PGHS-2) isoform. The subsequent conversion to the prostanoids (PGD₂, PGE₂, PGF_{2α}, PGI₂ and thromboxane A₂) from the

common precursor (PGH₂) is tissue specific and is determined by the presence of synthetases for each prostanoid in some tissues and not in others (reviewed by Smith et al., 1992; reviewed by Goetzl et al., 1995).

Cellular recognition and effects of lipid mediators are explained by the existence of corresponding receptor(s), which are thought to be G protein linked with seven transmembrane domains (reviewed by Cockcroft, 1994; Goetz et al., 1995; Coleman et al., 1995; annually updated by IUPHAR); we will only describe here the prostanoid receptors. These receptors have been characterised pharmacologically in many bioassays systems, using the naturally occurring prostanoids, agonists and antagonists; which have shown differences on ligand affinities and second messenger responses. Based on those studies prostaglandin receptors (termed P-receptors) were initially classified into five basic subtypes (DP, EP, FP, IP and TP). The first letters correspond to the type of natural prostanoid that is a major ligand, being at least ten times more potent than any of the other prostanoids. Thus a specific receptor has been proposed for each prostanoid (PGD, PGE, PGF, PGI and TxA₂) and named DP, EP, FP, IP and TP receptors respectively. The EP receptors were subclassified into at least 3 subtypes initially; termed EP₁, EP₂ and EP₃ (Coleman et al., 1990). These subtypes bind PGE₂ with similar affinity, but are coupled to different intracellular second messenger responses (increase in calcium, increase in cAMP and decrease in cAMP respectively). They also present differences in their sensitivity to PGE agonists and antagonists. A forth EP receptor subtype was subsequently identified (EP₄) which is also positively coupled with cAMP, like the EP₂ subtype, but it is differentiable from EP₂ because of its insensitivity to the agonist butaprost and its sensitivity to the antagonists AH22921 and AH23848B (Coleman et al., 1994; Coleman et al., 1995; IUPHAR, 1996).

Table 1.1

Prostanoid receptors classification

From Coleman et al., 1994 and the IUPHAR (1996)

PGE2 receptors here and the rest in next page.

Prostanoid receptors cDNA cloned in different species are also indicated. The cloning of cDNA for 6 receptors (EP1, EP2, EP3, FP, IP and TP) in the mouse has been reviewed by Narumiya et al. (1995), cDNA for the EP4 was later incorporated to the other 6 (reviewed by Ushikubi et al., 1995) and the DP receptor cDNA has been cloned recently (Hirata et al., 1995). (•EP2): There was an initial confusion because cDNAs were cloned in mouse and man wrongly named as the EP2, the true EP2 was later cloned in man (Regan et al., 1994) and the previously cloned receptors are now known to be in fact the EP4 (Katsuyama et al., 1995). The seven human receptors (EP1, EP2, EP3, EP4, FP, IP and TP) are reviewed by Abramovitz et al., (1995) and Pierce et al., (1995), except DP cDNA that was cloned later (Boie et al., 1995). Receptors cDNA cloning in other species has been reviewed by Ushikubi et al. (1995).

TYPE	potency order	SUBTYPE	LIGAND	AGONISTS	ANTAGONISTS	TRANSDUCTION SYSTEM	SPECIES CLONED
EP	E>F,I>D,T	EP1	PGE2	Iloprost 17-phenyl PGE2 Sulprostone	SC19220 AH6809	Calcium increase via Gq	mouse human
		EP2	PGE2	Butaprost AH13205 Misoprostol	None	cAMP increase via Gs	mouse human
		EP3	PGE2	Enprostil GR63799 Sulprostone Misoprostol	None	cAMP decrease via Gi PI turnover increase via Gq cAMP increase via Gs?	mouse human rat rabbit cow
		EP4	PGE2	None	AH23848 AH22921	cAMP increase via Gs	mouse human rat rabbit

Table 1.1 Classification of prostanoid receptors (part 1)

TYPE	potency order	SUBTYPE	LIGAND	AGONISTS	ANTAGONISTS	TRANSDUCTION SYSTEM	SPECIES CLONED
DP	D>>E>F>I,T		PGD2	BW245C ZK110841 RS93520	BWA868C	cAMP increase via Gs	human mouse
FP	F>D>E>I,T		PGF2a	Fluprostenol Latanoprost Cloprostenol Prostalene	None	PI turnover increase via Gq	mouse human, rat rabbit cow, sheep
P	I>>D,E,F>T		PGI2	Cicaprost Iloprost Octimibate	None	cAMP increase via Gs	mouse human rat
TP	T>>D,E,F,I		TXA2	U46619 STA2 I-BOP EP 011 U44069	SQ29548 GR32191 AH23848 EP 092 ICI 192605 L-655240 BAY u3405 S-145 BM13505	PI turnover increase via Gq	mouse human

Table 1.1. Classification of prostanoid receptors (part 2)

This classification has been validated with cDNA cloning of each type and subtype in mice and humans (see table 1.1). Cloning experiments have revealed further diversity within the EP₃R subtype, this receptor may exist in a variety of isoforms, resulting from alternative splicing at the C-terminus. Splicing was first reported in cattle (Namba et al., 1993), but it was shown later that it occurs in other species and that splicing may be species specific (i.e. not all the isoforms found have an homologue in other species) (Pierce et al., 1995a). The isoforms are functionally different because they couple with different G proteins or with the same proteins but with different efficiency; apart from a decrease in cAMP that was the original response recognised in the EP₃ subtype, other second messenger responses (increase in intracellular calcium and increase in cAMP) can be transduced by some of these EP₃ isoforms (reviewed by Coleman et al., 1995). The mouse isoforms have been found to be different not only at signalling but also in desensitisation and tissue distribution (Negishi et al., 1995).

1.9.2 Historical background of the discovery of prostanoids and their receptors with special emphasis on prostaglandin E₂ (PGE₂) and its receptors (EP receptors)

The information of this section has been obtained mainly from reviews by Robertson, 1986; Dusting and Stewart, 1990; Urich, 1990; Negishi et al., 1993; Coleman et al., 1990; Coleman et al, 1994.

The activity associated with prostaglandins (PGs) was first detected by two American gynaecologists in the 1930s, they observed that the human uterus contracts when exposed to human semen. This observation was followed by those of Goldblatt and von Euler in the early 1930s, and prostaglandins were named by the latter author after the prostate gland, which appeared to be the source in human semen. In the 1950s, the first

PGs, PGE₂ and PGF_{2α} were purified in 1957 and seminal fluid was shown to relax the cervix (reviewed by Najak and Hillier, 1970). During the next decade the remaining PGs were isolated and named alphabetically from PGA₂ to PGH₂. It also became clear that prostanoids are synthesised via the cyclo-oxygenase pathway in response to extracellular stimuli, they are then quickly released and act as local hormones in neighbouring cells. This was the first example of paracrine hormonal regulation in tissues. In the mid 1970s, thromboxane (TXA₂) and prostacyclin (PGI₂) were discovered and it became apparent that prostanoids have very diverse biological activities, which created excitement about the multiple possibilities of their therapeutic use. But this also implied proneness to side effects as another limitation to be added to their instability. Considerable progress was made in creating more stable analogues but not so much in reducing side effects. The use of PGE₂ in gynaecology for the induction of labour and termination of early pregnancy started in the late 1960s towards the beginning of the 1970s (reviewed by Keirse, 1990). The first indication was the induction of uterine contractions and later the ripening of the unfavourable cervix (reviewed by MacKenzie, 1981; Elwood et al., 1981). The initial approach to reduce side effects in the induction of cervical ripening by PGE₂ was to use local routes of administration (reviewed by MacKenzie, 1981).

There was a need to rationalise the multiplicity and ubiquitous distribution of prostanoids actions and this started with work on their binding sites in the 1970s. Kuehl and Humes (1972) showed the first evidence for the existence of prostaglandin E receptors in rat lipocytes (reviewed by Negishi et al., 1993). This was followed by other binding studies, first with [³H] PGs E₁, E₂ and F_{2α} and later with [³H] PGs D₂ and I₂. These studies showed high affinity (K_d: 1-10 nM) and density (1 pmol/mg protein) in a wide range of tissues, namely: lipocytes, liver, adrenal cortex, adrenal medulla, liver, ovary, uterus, kidney, stomach, ileum, thymus, skin,

brain, lung and blood cells. These findings supported but did not demonstrate the existence of functional receptors in those tissues. But later, it became clear that some of these binding sites represent functional receptors, with the demonstration in the early 1980s, that they can be up and down-regulated by exposure to the ligand or inhibitors. Binding site studies continued until the present, but in most cases using isolated cells and analogues that displace the binding, enabling the differentiation between receptor subtypes (Nishigaki et al., 1993; Wise and Jones, 1994).

The development of synthetic prostanoid agonists and antagonists allowed the first classification of prostanoid receptors into types for each individual prostanoid (i.e. DP, EP, FP, IP and TP) by Kennedy and colleagues in 1982 (reviewed by Coleman et al., 1994). Some analogues also presented therapeutic advantages and were used for cervical ripening, being more specific for uterine tissues and having a longer half life (recently reviewed by Bygdeman, 1992). In the 1980s, intracellular second messenger studies provided further evidence for the existence of prostanoid receptors and revealed the existence of subtypes of EP receptors. Prostaglandins of the E series caused increase or decrease in cAMP and each of these responses was accompanied with distinct and some times opposing functions in platelets and other cells (reviewed by Coleman et al., 1990; Coleman et al., 1994). Furthermore, in the 1990s a third pathway, the inositol triphosphate (IP3), has been discovered in bovine adrenal chromaffin cells (reviewed by Negishi, 1993).

There have been difficulties in the solubilization and purification of prostanoid functional receptors, which has retarded the molecular characterisation of these receptors. The first attempts to solubilize prelabelled PGE₂ and PGF₂ α receptors in the mid 1970s were unsuccessful, finding that the activity was lost and that the receptors were only stable in the presence of the ligand. From the mid 1980s, partial

solubilization and purification of active PGE₂ receptors was achieved from several tissues: porcine cerebral cortex, bovine adrenal medulla, canine renal medulla and murine macrophage (Yumoto et al., 1986; Negishi et al., 1993; Fernández-Botran and Suzuki, 1984). The estimated molecular weights of those receptors ranged from 95- 110 KDa, with the exception of one study, where the canine renal medulla PGE₂ receptor was reported to be lighter (65 KDa). The solubilization aimed at the purification to further characterise these proteins and their sequences, but there were problems of low content in detergent extracts compared to other receptors, instability and loss of function during purification (Michalack et al., 1992; reviewed by Negishi et al., 1993). The human thromboxane A₂ receptor (hTPR) is the only prostanoid receptor that has been purified so far. It was also the first eicosanoid receptor to be purified, this was done from human blood platelets, taking advantage of the development of highly potent antagonists and their high affinity radiolabelled derivatives (Ushikubi et al., 1989). A PGE₂ functional receptor was later purified from cardiac sarcolemma (Michalack et al., 1992) but we are unaware of further characterisation of these receptors.

The first cDNA cloned for an eicosanoid receptor was also the human thromboxane A₂ receptor, based on the partial amino acid sequence of the recently purified protein (Hirata et al., 1991). Hydrophobicity analysis of the deduced amino acid sequence and its comparison with other rhodopsin-type receptors, led to the conclusion that this receptor was a G-protein coupled receptor belonging to the rhodopsin-type family. The hypothesis that the TP receptor could belong to a new group of receptors contributed to the subsequent cloning of the prostaglandin E₂ receptor subtype (EP₃) in the mouse, based on homology screening of cDNA libraries (Sugimoto et al., 1991; Sugimoto et al., 1992). The knowledge of those sequences has allowed the cloning of cDNA for

the rest of the prostanoid receptors group in those species (mouse: Hirata et al., 1994; reviewed by Narumiya et al., 1995; Katsuyama et al., 1995 and humans: reviewed by Abramovitz, et al., 1995; Pierce et al., 1995a; Boie et al., 1995). Gradually many of these receptors cDNA are being cloned within several mammalian species (rat, rabbit, bovine and ovine) (see table 1.1) (reviewed by Negishi et al., 1995; reviewed by Ushikubi et al., 1995). Cloning has shown that subtypes exist for the EP₃ receptor in all the species studied and probably in the TP receptor too (reviewed by Negishi et al., 1995).

At the beginning of this studies, the only prostanoid receptor and gene to be fully characterised was the human TPR (Nürsing et al., 1993). For other prostanoid receptors, the only sequence information available was deducted from cDNAs cloned in a limited number of mammalian species, mainly the human and the mouse. Therefore the number of reagents available (probes and antibodies) for the study of most prostanoid receptors was scant. Antibodies for the EP receptors were beginning to be developed at the time of the writing of this thesis based on the deducted amino acid sequences from the cDNA sequences (Fujimoto et al., 1995). The gene characterisation is still in progress for most prostanoid receptor types and subtypes (see next section 1.9.3: gene structure, page 87).

1.9.3 Molecular biology of the EP receptors and other prostanoid receptors

Structure of the proteins and its corresponding mRNAs

The overall homology of the prostanoid receptors with other rhodopsin-type G-protein receptors is low, they share 10-20% homology with adrenergic and muscarinic receptors. However, several amino acids (of recognised importance for the structure and function of rhodopsin-type receptors) are conserved in the prostanoid receptors (reviewed by Ushikubi et al., 1995).

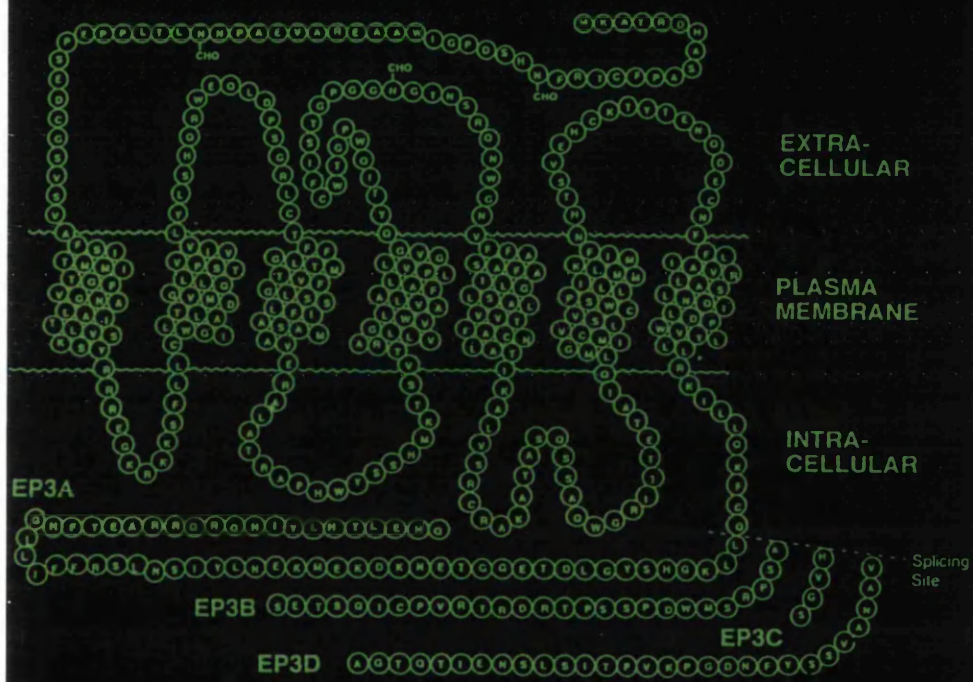
The overall homology between prostanoid receptors is not very high either (20-30%) and is scattered in the entire sequence, which suggests the prostanoid receptors did not evolve from a common gene (Coleman et al., 1994). The gene localisation in several chromosomes, also supports this view (Taketo et al., 1994; Duncan et al., 1995). The homology, however, is higher between receptors of similar functions and three groups have been defined: 1) the contractile receptors (TP, FP and EP₁) which mobilise calcium and share 47-48% identity at the transmembrane (TM) region, 2) the relaxant receptors (DP, IP, EP₂ and EP₄) positively coupled with cAMP and sharing 39-44% overall homology in the entire sequence and finally 3) the inhibitory EP₃ receptor, which is less than 30% homologous with other groups. Supporting this argument, the homology between the EP subtypes, belonging to a different functional group, is lower than 32% (reviewed by Narumiya, 1995; Ushikubi et al., 1995).

Plate 1.3

Diagram of seven transmembrane domain model of the bovine EP3 receptor, showing alternative splicing at the C terminal.

(From Coleman et al., 1994)

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There are two highly conserved areas between all the prostanoid receptors: 1) mainly a sequence in the VII TM domain (LAXAXRXAS/TXNQILDPWVYILR), 2) but also to a lesser extent, two sequences in the 2nd extracellular loop (GRYXXQXPGT/SWCF and MXFFGLXXLLXXXAMAXER). Therefore it is also possible that they could have evolved from a common ancestor gene (Coleman et al., 1994; Narumiya, 1995; Pierce et al., 1995a; Ushikubi et al., 1995). These areas are unique to the prostanoid receptors and conserved between different species, therefore they may participate in ligand binding. Furthermore, all the prostanoid receptors have an arginine in the VII TM domain (position 295 in the TXA₂ receptor) which has been demonstrated to have a role in ligand binding activity (reviewed by Ushikubi et al., 1995).

The identity between sequences of cDNAs encoding for receptor homologues from different species is high; ranging from 79-89% between the human and mouse receptors and from 84-97% between the EP₃ receptors in different mammalian species (human, bovine, rat and mouse). Despite this high homology, there are functional differences between species. Different sizes have also been observed between receptor homologues of different species, due to differences in the translation initiation site (reviewed by Ushikubi et al., 1995).

Distribution of the prostanoid receptors and their mRNAs

The distribution of these proteins has been studied in biochemical and pharmacological studies, which have shown their ubiquitous expression in the body and some of their biological actions (Robertson et al., 1986; Coleman et al., 1990; Rao, 1990; Negishi et al., 1993). But these type of studies suffered from lack of specificity and sensitivity. The cloning of the receptors cDNA allows better characterisation of the distribution of these receptors using northern analysis, reverse transcription and polymerase chain reaction (RT-PCR) and in situ hybridisation (ISH). In addition, in the absence of amino acid sequences from protein studies for most prostanoid receptors, antibodies may be developed from the deduced amino acid sequences from the cDNAs, to study the proteins distribution. These type of studies have been initiated in the eye (Fujimoto et al., 1995).

Molecular biology studies have shown in recent studies that each prostanoid type (Coleman et al., 1994; Abramovitz et al., 1995; Narumiya et al., 1995) and subtype (Negishi et al., 1995) has a characteristic pattern of mRNA expression in tissues and that expression levels are variable among tissues. This pattern is conserved between species to a certain extent only, some receptors present in a particular tissue of one species are absent in the same tissue of other species (reviewed by Ushikubi et al., 1995). Similarly, the localisation of receptors within the kidney may be variable between species. The mRNA tissue distribution in most cases is consistent with previous studies of the protein, but new localisations and functions have also been discovered with this approach (reviewed by Ushikubi et al., 1995).

Gene localisation of the prostanoid receptors and structure

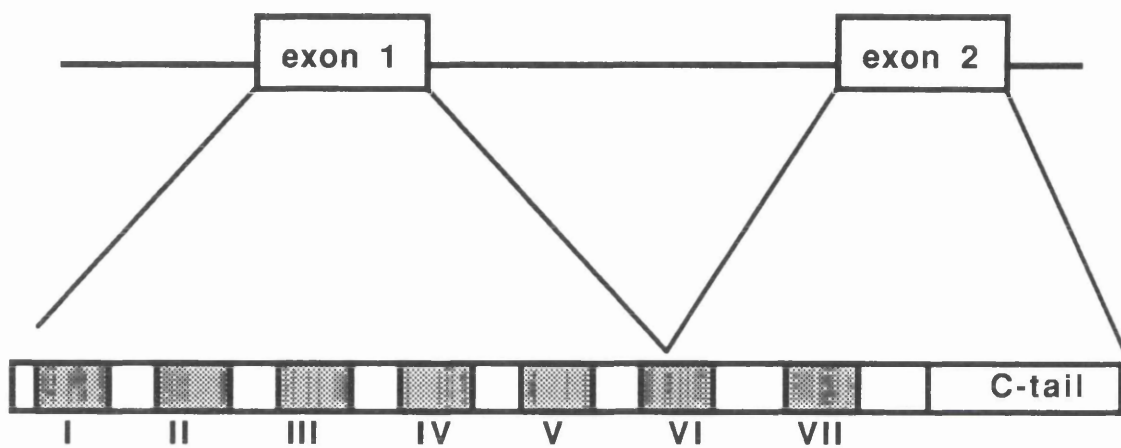
The genes for some of these receptors are located in three chromosomes; in the mouse these are chromosome 3 (EP₃), 10 (TP) and 15 (EP₄) (Taketo et al., 1994) and in humans chromosomes 1 (FP and EP₃ receptors), 5 (EP₂ R) and 19 (EP₁, TP and IP receptors) (Nürsing et al., 1993; Duncan et al., 1995). In the rat, the gene encoding for the EP₃ receptor has been located in the chromosome 2 (Takeuchi et al., 1996).

At the beginning of these studies the gene structures of the prostanoid receptors were unknown. It is not surprising that the first gene to be cloned was also the human thromboxane A₂ (Nürsing et al., 1993), as with protein isolation and cDNA cloning. The human TXA₂ receptor gene is present as a single copy, spans over 15 kilobases and contains 3 exons divided by 2 introns. The larger intron (6.3 kb) is located in the 5'-noncoding region and a shorter one (4.3 kb long) can be found at the end of the region encoding for the VI transmembrane domain (VI TM). Two promoters have been found that lead to alternative splicing of exon 1, it is unclear whether isoforms are produced as a result or not. Some authors have reported the production of only one type of protein (Nürsing et al., 1993), whereas others have found two proteins (Kinsella et al., 1995). Others have found two TP isoforms with different cytoplasmic tails created by the presence or absence of a part of exon 3 (Raychowdhury et al., 1994).

Figure 1.1

Gene structure of the mouse DP receptor

From Hirata et al., (1994). The open reading frame consists of two exons (open boxes) separated by an intron. The localisation of an exon/intron splice site at the end of the VI TM is also present in the EP₁ and TP receptor genes and it may be conserved in all prostanoid receptor genes.



The next gene to be cloned was the mouse DP receptor (Hirata et al., 1994). The structure is similar to the human TP receptor but may be simpler, since no alternative splicing has been identified. The open reading frame in the mouse DP receptor, also consist of two exons separated by an intron at a homologous position (end of the VI TM) to the human TP receptor. Observations of the genes of other prostanoid receptors (EP₃: Regan et al., 1994a; EP₂: Regan et al., 1994b) suggested that the intron at the VI TM region could be a common feature of this receptors' group. Recent cloning of the gene for human DP (Boie et al., 1995) and the mouse EP₁ receptor (Bátshake et al., 1995) confirm that the intron position and the basic structure of two exons are conserved.

In common with the other prostanoid receptors, several authors are confirming the initial observations of an intron at the VI TM region in the human (Regan et al., 1994a; An et al., 1994) and in rabbit EP₃ receptors (Breyer et al., 1994; Tarng and Breyer, 1995). The EP₃ receptor however, appears to have a more complicated and long gene structure than other prostanoid receptors, presenting multiple fashions of alternative splicing in the area encoding for the C-terminal end, which has been reported in all the species studied (Namba et al., 1993; Sugimoto et al., 1993; Takeuchi et al., 1993; Breyer et al., 1994). Several and different exons encode for the C-terminal in different species. In the rabbit, at least 17 exons encoding for the C-terminal of the EP₃ isoforms may exist (Tarng and Breyer, 1995).

1.9.4 PGE₂ receptors and other prostanoid receptors in uterine tissues: myometrium, endometrium and cervix uteri

PGE₂ and other prostanoids participate in several functions of the uterine tissues of mammals: myometrial contractions, implantation of the embryo in the endometrium and cervical ripening. The uterus is contemplated here as having three compartments: the myometrium, endometrium, and the cervix;

because they differ in their responses to prostanoids (reviewed by Rao, 1990; Coleman et al., 1990). The prostanoid receptors have been more extensively studied in the myometrial than in the endometrial and cervical functions (reviewed by Rao, 1990). Binding sites for PGE₂ have been found in the three uterine tissues (Rao, 1990).

Binding site studies of eicosanoid receptors in the myometrium started early in the 1970s (reviewed by Rao, 1990). These studies suggested that the affinity of prostaglandin receptors was greater for PGE₂ than for FGF₂ α , which was consistent with the higher potency of exogenous PGE₂ than PGF₂ α at inducing uterine contractions (Giannopoulos et al., 1985; Adelantado et al., 1988). Studies of cellular distribution of binding sites found EP receptors in the myometrial smooth muscle, stromal cells, glandular epithelium, arterioles and erythrocytes (Chegini et al., 1986; reviewed by Rao, 1990). Second messenger studies initiated in the 1980s, established that increases in intracellular calcium levels promote contraction, whereas cyclic nucleotides (cAMP and cGMP) promote relaxation of the myometrium. Pharmacological studies using agonists and antagonists have shown which prostanoid receptor type or subtype exist in different species and species differences (reviewed by Coleman et al., 1990). These studies have been expanded recently in the sheep (Garcia Villar et al., 1993; Garcia Villar et al., 1995; Crankshaw and Gaspar, 1995). Some receptors (DP, EP₂, EP₄ and IP) mediate relaxation of the myometrium, whereas other receptors (EP₁, EP₃, FP and TP) mediate the contractions. All of these studies provide strong support for the existence of certain prostanoid receptors in the myometrium. Abundant mRNA expression of all the prostanoid receptors has been detected by northern blots in the mouse and human uterus (Abramovitz et al., 1995; Narumiya et al., 1995) and by RT-PCR in the human myometrium (Senchina and Crankshaw, 1995). Several uterine cDNA libraries have been used to clone

some of these receptors.

Prostaglandin receptors in the endometrium are independently regulated from those in the myometrium (reviewed by Rao, 1990). PGE₂ and EP receptors are involved in the uterine receptivity to the blastocyst implantation in several species (laboratory species, man and pig), which is time and hormone-dependent and the presence of the receptors is also regulated in this fashion (reviewed by Rao et al., 1990). It is interesting that in species like sheep and cattle, the endometrium produces PGE₂, before implantation, during maternal recognition and some authors think that this PG does not act on the endometrium, but on the rescue of the *corpus luteum*, which presents increased numbers of binding sites at that stage (Wiepz et al., 1994).

Studies of prostanoid receptors in the cervix are limited to only a few binding sites and second messenger studies. Binding studies for PGE₂ and PGF_{2α} in the cervix are not very detailed because they are also aimed at the study of the myometrial function. Results have been variable, EP receptors were either undetectable (Bauknecht et al., 1981) or of a lower density (Hoffman et al., 1983; Giannopoulos et al., 1985) and/or affinity (Adelantado et al., 1988) in the human cervix than in the myometrium. The low density found in the cervix and the high density found in the uterus were highly correlated with the smooth muscle content in those two tissues (Hoffman et al., 1983; Giannopoulos et al., 1985). The endometrium was scraped from the uterine proper samples, but it is unclear whether the endocervix was included in those studies or not. The binding sites detected in the cervix may or may not represent functional receptors, it is not known whether the receptors can be up or down regulated by the ligand and regulation by steroids was not obvious either. The existence of subtypes has not been investigated with the use of agonists and antagonists either (Coleman et al., 1994). The cervix responded to PGE₂ with an increase in

the levels of intracellular cAMP (Norström et al., 1983; Norström and Bryman, 1991). The second messenger cAMP, like in the myometrium, appears to be involved in the inhibition of spontaneous contractions by the cervix (Norström and Bryman, 1991).

But many authors believe that PGE₂ causes cervical ripening by promoting the degradation of the connective tissue rather than acting on cervical contractility (see chapter I). In this sense, although PGE₂ and cAMP influence collagen metabolism (determined by hydroxyproline incorporation) (Norström et al., 1983), other more recent studies have shown that cAMP inhibited the expression of the proMMP-1 and MMP-3 by cervical fibroblasts (Takahashi et al., 1991). Therefore the role of this pathway (which may involve the EP₂ or the EP₄ receptors) in the remodelling by metalloproteinases during cervical ripening is unknown. An increase of cAMP was considered the typical response of EP₂ or EP₄ receptors for some time, but recent studies have shown that the EP₃R may mediate increases of cAMP in some tissues too (Irie et al., 1993). Other pathways that may involve increases in calcium and the other receptors subtypes (EP₁ and EP₃) have not been investigated in the cervix uteri. But, the administration of PGF_{2α}, IL-1 and protein kinase C (PKC) to cervical tissue cultures induced an increase in collagenase activity, furthermore PKC appears to mediate the effect of PGF_{2α} and IL-1 (Rajabi et al., 1992; Takahashi et al., 1993). Rajabi and colleagues (1992) have suggested, but not demonstrated, a pathway involving FP receptors (through Ins P₃, PCK and an increase in calcium) in the induction of proMMP-1 synthesis by fibroblasts. They also speculate about PGE₂ increasing collagenase activity by being converted into PGF_{2α}.

CHAPTER 2

MATERIALS AND METHODS

2.1 MICROSCOPIC STUDIES

2.1.1 Light microscopy (LM)

i) Fixation

Cubes of 1 cm wide were dissected and fixed for 1 week in 4% buffered neutral formalin pH: 7.2 (BNF).

BNF

100 ml/l formalin (40% w/v)

4 gr/l sodium dihydrogen ortophosphate

6.5 gr/l disodium hydrogen ortophosphate

It is made up in tab water.

ii) Processing cycle

The following procedure is for processing tissue for embedding in paraffin wax. Fixed tissues were placed in cassettes which were loaded in a basket of the Shandon Elliot automatic tissue processor (Histokinette) and processed through the following solutions:

- 1) 70% alcohol for 1 hour and 30 minutes
- 2) methylated spirits for 1 hour and 30 minutes
- 3) methylated spirits for 2 hours
- 4) methylated spirits for 3 hours
- 5) absolute alcohol for 3 hours
- 6) absolute alcohol for 2 hours
- 7) absolute alcohol for 1 hour
- 8) alcohol - chloroform (50:50) for 1 hour
- 9) chloroform - histoclear (50:50) for 2 hours
- 10) histoclear for 1 hour and 30 minutes
- 11) paraffin wax for 3 hours
- 12) paraffin wax for 2 hours and 30 minutes

The cycle takes 24 hours in total.

iii) Embedding in wax and sections

The processed tissues were blocked out in 60°C paraffin wax, sections were cut at 3-5 µm with a Leitz rotary microtome and mounted on glass slides.

iv) Staining Procedures

Mounted sections were stained with 3 standard methods: Haematoxylin and Eosin (H&E), Periodic acid schiff (PAS) and Alcian blue/ PAS (AB/PAS). Two specific methods for the connective tissue were also used: Masson's trichrome and Martius scarlet blue (MSB).

2.1.2 Transmission electron microscopy (TEM)

i) Fixation

Tissue was minced in Karnovsky's fixative (into less than 1 mm³) in a Petry dish with a razor blade. Then transferred into fresh fixative and left for a minimum of 3 hours or overnight at room temperature. The fixative was then drained and replaced with 0.1 M Cacodylate buffer for a minimum of 1 hour or overnight. Tissue was post-fixed in a fume cupboard in 1 % osmium tetroxide. Finally tissue was washed in distilled water.

Modified Karnovsky's fixative

2 % paraformaldehyde

2.5 % glutaraldehyde

Made up in 0.1 M sodium cacodylate buffer.

10 gr of paraformaldehyde were dissolved in 100 ml of distilled water, add 10 drops of 1 N NaOH, cover and heat it in a 60°C oven to dissolve. When cool this mixture was added to the stock solution.

stock solution

0.2 M cacodylate buffer	250 ml
2.5% glutaraldehyde	50 ml
distilled water	100 ml

2.5 % glutaraldehyde (for 1 litre of fixative)

21.4 gr of sodium cacodylate
40 ml of 0.2 M HCl
100 ml of 25 % glutaraldehyde
660 ml of water

0.2 M sodium cacodylate buffer (500 ml)

250 ml of 0.4 M sodium cacodylate (21.4 gr/250 ml)
40 ml of 0.2 M hydrochloric acid (HCl)
210 ml distilled water

ii) Resin processing

All stages of the processing were performed in a fume cupboard.

NB: Resin kit (Emix kit medium, Fisons Scientific Equipment, Loughborough) was used which is made up by the following components:

25 ml of emix resin: low viscosity modified bisphenol A epichlorohydrin epoxide; 25 ml of hardener: mixture of several anhydrides coupled with a plasticizer, dodecenyl succinic anhydride (DDSA) and 1 ml of accelerator: the tertiary amine benzylmethylamine. These component are mixed with a mechanical stirrer for about 1 hour before used, and can be stored in the freezer at -20°C.

Dehydration in a graded series of acetone in water

The distilled water was then drained and replaced with 70 % acetone for 10 minutes or overnight, 90 % for 10 minutes and 100 % two times for 15 minutes.

Clearing

The 100 % acetone was then drained and replaced twice with the clearing agent propylene oxide for 20 minutes.

Infiltration with resin

Emix resin was added to the last propylene oxide (50:50) and mixed in a Taab rotor (20 rpm) for 1 hour. This mixture, was drained and replaced by emix resin that was left rotating for 3 hours.

iii) Embedding

Processed samples were then embedded in beam capsules filled with resin mix and were left to polymerize in a 60°C oven for 2 days.

iv) Thick sections

Thick sections of 1 µm were cut from each block using an LKB Pyramitome and after flattening with xylene vapour, stained with Toluidine blue.

Toluidine blue stain

1% borax (sodium tetraborate) 1 gr

1% toluidine blue 1 gr

distilled water 100 ml

staining time was 15 secs.

After examination in a light microscope (Leitz Laborlux II), suitable areas were selected to trim the EM blocks.

v) Ultrathin sections

Ultrathin sections in the silver or pale gold range (60-90 nm thick) were cut in a LKB MK III ultramicrotome, flattened using xylen vapour and picked up on gold mesh grids (Polaron 300). Sections were stained with uranyl acetate and lead citrate and examined in a Hitachi HS8 electron microscope. Electron micrographs were taken using Ilford Technical EM plates, developed in PQ Universal and fixed in Ilford Ilfodspeed fixer.

Uranyl acetate

A saturated solution was prepared by dissolving 0.2 gr. of uranyl acetate in 10 ml of distilled water.

Staining time was 5 min.

Lead citrate

1.33 g. of lead citrate and 1.76 of sodium citrate were dissolved in 30 ml of distilled water, shaking for 30 minutes. 8 ml of 0.1 M NaOH and distilled water were added up to a final volume of 50 ml.

Staining time was 5 min.

2.1.3 Scanning electron microscopy (SEM)

i) Fixation

Before fixing the tissue was washed in buffer to remove surface blood and mucous. Samples of tissue were immersed in chilled Karnovsky's fixative for a minimum of 24 hours. Tissue was then trimmed to expose surface to be scanned and washed in 0.2 M cacodylate buffer for 4 hours.

ii) Dehydration in a graded series of acetone in water

70 % for 4 hours or overnight

90 % for 2 hours

100 % for 2 hours

100 % overnight

iii) Critical point dry

Using CO₂ in a critical point drier (Biorad machine)

iv) Mounting

Specimens were mounted in aluminium stubs using silver paint and left to dry in a 60°C oven.

v) Sputter coating with gold/palladium

Using an EM Scope SC 500 sputter coater for 4 minutes.

vi) Scanning and prints

Specimens were examined in a Philips 501 B scanning electron microscope operating at 15 Kv. An automatic Rolliflex camera and Ilford FP4 120 (125 ASA) film were used.

2.2 MOLECULAR BIOLOGY STUDIES

2.2.1 Animals and tissue collection

Mouse specimens were obtained from male and female adult animals bred in the animal house of the Glasgow Veterinary School, they were sacrificed by cervical dislocation.

Sheep specimens were obtained from various sources:

Cervix of periparturient (n=10, 143 and 144 day's gestation) and of luteal phase sheep (n=10, days 9, 10, 12 and 13 post oestrus) were kindly supplied by Dr. Elizabeth Scott at The Moredun Research Institute and by Dr. Ian Wilmut at Roslin respectively. In both cases the oestrous cycle of the ewes was synchronised with progestagen sponges and the reproductive stage closely monitored for their own experiments.

Early luteal phase cervix (day 6 after oestrus) and oestrus cervix (two days after sponge removal) were collected at the Anatomy Department from sheep (n=4) whose cycle had been previously synchronised with progestagen sponges containing 60 mg of medroxyprogesterone acetate (Veramix, Upjohn). The reproductive status was confirmed by ultrasonography of the reproductive tract in these animals (see chapter V for more details). Blood samples were collected from all the mentioned ewes, plasma was separated and stored frozen to confirm the reproductive status with steroid levels (progesterone and oestradiol), if needed.

The other ovine specimens (kidney, uterus, spleen, liver, testes and adrenal gland) were collected from either the animals mentioned earlier, the post-mortem room in the Veterinary School or from the local abattoir. From all tissues, samples of less than 1 gram each, of approximately 1 cm³ in size, were rapidly dissected, frozen in liquid nitrogen and stored at -70°C until the extraction of total RNA was carried out.

2.2.2 RNA extractions

Total RNA was prepared from individual tissues using the acid-guanidinium-phenol-chloroform method (AGPC) described by Chomczynski and Sacchi (1987) or its recent modification (Chomczynski, 1993). Briefly, frozen tissues were placed in sterile tubes with 1 ml of a commercial isolation reagent, RNazol B (Cinna/Biotech), in most cases or TRIzol (Gibco) with a few samples. Tissues were defrosted in the buffer and then homogenised using an ultraturrax. The isolation reagents, have potent deproteinizing agents like guanidium-thiocyanate, phenol (RNazol B and TRIzol) and mercaptoethanol (RNazol B), which inactivate RNases that are liberated from the mechanical shearing of the tissue during homogenisation and could otherwise degrade RNA. They also contain solubilizing agents for RNA, which allow separation from DNA, proteins, etc. After the homogenisation, RNA was pelleted and purified according to manufacturers' instructions with slight modifications. To extract the RNA, the homogenate was transferred into a sterile eppendorf tube and 1/10 of its volume in chloroform was added and mixed with a vortex for 15 seconds and left to set for another 5 minutes on ice (RNazol method) or room temperature (Trizol method). This mixture was then centrifuged at 12000g for 5 minutes, which resulted in separation of three phases; a colourless aqueous phase containing the RNA on top, a DNA and proteins interface and the lower phenol-chloroform phase (blue for RNazol or red for Trizol). From this point all the efforts were put in maintaining RNase-free conditions (use of gloves, use of sterile plastic ware, use of filter tips) when handling the RNA. The aqueous phase was transferred to a fresh tube and RNA was then pelleted by precipitation with alcohols as follows. The first precipitation was carried out by adding an equal volume of isopropanol, storing the samples for 1 hour at 4°C (RNazol) or for 10 minutes at room temperature

(Trizol) and centrifuging at 12000 g for 15 minutes. A white-yellow pellet forms at the bottom of the tube that is washed in ethanol; by removing the supernatant, adding 75% ethanol, vortexing and centrifuging gently (at 7500 g for 8 minutes). In the case of the RNAzol method a second precipitation in the presence of NaAc (3M, pH=7) and absolute ethanol was added to the original protocol. The 75% ethanol is removed and RNA is then solubilized in 100 µl of PCR water and mixed with 1/10 volume of sodium acetate and 2 volumes of absolute ethanol at -20°C. The mixture is stored for 1 to 24 hours at -20°C and then centrifuged at 12000 g for 10 minutes. In both cases, at the end of the procedure, ethanol is removed with a filtered tip and a rolled tissue and the RNA pellet is then dissolved in 20 µl of PCR water by vortexing, passing through a pipette tip and if necessary by incubating at 60°C for 10 minutes before storing at -70°C.

The integrity of the RNA was ascertained by running 1.5 µl of the resuspended RNA in a non denaturing 1% agarose gel. Ribosomal RNA (rRNA) constitutes most of the total RNA and should be clearly visible with the correct mobility. The observation of two bright ribosomal bands (28 S and 18 S), corresponding to approximately 5.1 kb and 1.9 kb in eukaryotes, is therefore indicative of nondegraded RNA. On the contrary, when RNA is degraded it has smaller molecular weight and higher mobility and the rRNA is not clearly visible. Smaller molecular weight RNA (0,1-0,5 kb) is observed as a smear or as a bright band at the bottom of the gel (Chomczynski, 1993, Darling and Brickell, 1994).

2.2.3 Deoxyribonuclease I (DNase I) incubations

Before the RT-PCRs for the study of mRNA expression, RNA samples were treated with RNase-free DNase I (from bovine pancreas) (Stratagene) to remove contaminating genomic DNA (Grillo and Margolis). RNA (approximately 5 µg) was incubated at 37°C for 30 minutes under the following conditions: 40 mM RNase free Tris-HCl pH 7.6 (Sigma), 6 mM Mg Cl₂ (Advanced Biotechnologies, filtered through pore size 0.2 µm), 1.6 units/µl of RNase inhibitor, (RNasin, Promega) and 1 unit of DNase I (Stratagene) in a final volume of 25 µl. The reaction was then heated at 95°C for 5 minutes to inactivate the DNase I, immediately cooled on ice and used in the cDNA synthesis.

The following controls were done to establish the above conditions:

i) Residual RNase activity in the enzyme DNase I (Stratagene) or the rest of the individual reaction components.

The integrity of the DNase-treated RNA was evaluated by electrophoresis in 1% agarose gels.

ii) DNase I activity

Evaluated by electrophoresis in 1% agarose gels of the digestion of molecular weight markers 100 bp ladder (Gibco).

iii) Minus RT controls to monitor for the removal of DNA from RNA samples

2.2.4 RT-PCR

An RT-PCR method was used to isolate a cDNA fragment of the EP₃ receptor. The product was identified by sequencing and Southern blot. The mRNA expression of the EP₃ receptor was studied by northern blot analysis and RT-PCR. A house keeping gene was used as a positive control of the integrity of the RNA and also to control for the variability in the RT performance. Work was also done in an attempt to develop a

semiquantitative RT-PCR assay, comparing the levels of EP₃R mRNA expression relative to β -actin between different samples.

i) cDNA synthesis by Reverse Transcription (RT)

mRNA was reverse transcribed into single stranded cDNA from total RNA with Moloney Murine Leukemia Virus (RNase H⁻) Reverse Transcriptase (SuperScript, Gibco) in the presence of random hexamers as published by others with slight modifications (O'Shaughnessy and Murphy, 1993).

Briefly, RNA template (300 μ g/ μ l, approximately 0.06-2 μ g) was added to a reaction mixture consisting of: 50 mM KCl, 10 mM Tris-HCl pH 8.3 (at 25°C), 3 mM MgCl₂ (Advanced Biotechnologies Ltd.), 1 mM dithiothreitol (DTT), 5 μ M random hexamers, 500 μ M of each dNTP, 6 units/10 μ l RNase block (rRNasin, Promega) and 3 units/ μ l M-MLTV reverse transcriptase (SuperScript RNase H⁻, Gibco). The buffer (KCl, Tris-HCl and MgCl₂), water and DTT were previously treated with UV light for 5 minutes. The reaction was incubated at room temperature for 5 minutes then at 37°C for 1 hour. The cDNA synthesised was stored at -20°C until used as template for the PCR reaction.

ii) Cross species PCR for the isolation of a cDNA fragment encoding for the EP₃ receptor and mRNA expression studies

The isolation and sequencing of an ovine prostaglandin E₂ receptor subtype (EP₃R) cDNA fragment was done by cross-species RT-PCR, using two bovine oligonucleotide primers. Primers were designed from a published mRNA bovine sequence of the EP₃ receptor (Namba et al., 1993) at regions highly conserved between the species published at the time (human: Adam et al., 1994; rat: Takeuchi et al., 1993; mouse: Sugimoto et al., 1993, and rabbit: Breyer et al., 1994). Minimal degeneration in the code

was also aimed, specially at the 3'end, to minimise mismatches between the bovine primers and the ovine template. Relatively long primers (25 bases long) were chosen, because this has been reported to be more effective when amplifying homologous genes from a different species (Dieffenbach et al., 1993). The selected primers were checked for absence of homology with other sequences of mammalian species stored in the EMBL Gene Bank, especially at the 3' prime end, using the program GCG.

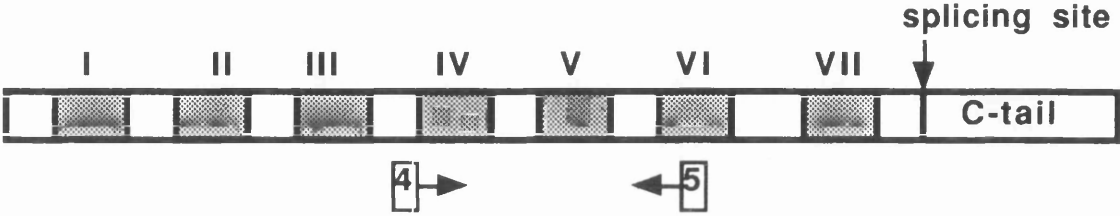
Apart from those special considerations for cross species PCR, the rest of the general criteria in the design of PCR primers (optimal A/T: G/C ratio, composition of less than 60% purines, lack of complementarity between primer pairs, similar melting temperatures of both primers) were also followed; except for the requirement of primers to span one intron, since the structure of the gene (intron and exon boundaries) was not published for this gene in any species at this time. Primers were also chosen to amplify a region larger than 300 bp, since exons greater than that size are rare in vertebrates (Gause and Adamovicz, 1994). Bovine EP₃ receptor primers amplified cDNA encoding a fragment of the bovine protein between the 2nd intracellular loop to the VI TM region and the size of the expected product was 355 bp (from nucleotide 481 to 835 inclusive) (figure 2.1). The sequences and the nucleotide positions of the sense (P4, Oligo Express Ltd) and antisense primer (P5, British Biotechnology Products) are indicated in table 2.1 (second set of primers).

Numbers	Names	nucleotides	Sequence (5' to 3')	Size of product(s)
P1	RACE (RT)		CTC GAG GTC GAC GGT ATC GAT ATT TTT TTT TTT TTT TTT	
P2	RACE (ADAPTER)		CTC GAG GTC GAC GGT ATC GAT A	947-1037 bp
P3	5'end	236-256	GGG AGG GCA AGC GCA AGA AG	
P5	3'end	811-835	TCT CTG TCG TGA TTC GGC CCC ACTG	355 bp
P4	5'end	481-515	CCG CAC TGG TAC TCA AGC CAC ATGA	
P1	RACE (RT)		CTC GAG GTC GAC GGT ATC GAT ATT TTT TTT TTT TTT TTT	
P2	RACE (ADAPTER)		CTC GAG GTC GAC GGT ATC GAT A	647-812 bp
P4	5'end	481-515	CCG CAC TGG TAC TCA AGC CAC ATGA	
P7	RACE (RT)		GAC TCG AGT CGA CAT CGA TTT TTT TTT TTT TTT TT	
P6	RACE (ADAPTER)		GAC TCG AGT CGA CAT CG	718-808 bp
P4	5'end	481-515	CCG CAC TGG TAC TCA AGC CAC ATGA	

Table 2.1: primers used to amplify EP3R cDNA

Figure 2.1

Position of the PCR primers in the cDNA bovine sequence. Sense primer (4) is located in the region encoding for the 2nd intracellular loop, between the III and the IV transmembrane (TM) domains. The antisense primer (5) is located in the VI TM domain. The size of the expected product is 355 bp.



Initially, low stringency conditions were used in the PCR for the isolation and sequencing of a cDNA fragment. The PCR reaction mixture consisted of: 20 mM $(\text{NH}_4)_2 \text{SO}_4$, 75 mM Tris-HCl pH 9.0 (at 25°C) 0.01% (w/v) Tween, 3 mM MgCl_2 (Advanced Biotechnologies Ltd.), 400 nM of each primer, 200 μM of each dNTPs, 8.3 units/ml Taq DNA polymerase (Advanced Biotechnologies Ltd.) and 2 μl of cDNA template in a final volume of 30 μl . The buffer, MgCl_2 and H_2O were previously treated with UV light for 5 minutes. Mineral oil (30 μl) was overlaid and the reaction began with an incubation at 95°C for 3 minutes, followed by 32 cycles of: denaturation of double strand (94°C for 20 seconds), primer annealing (50-55°C for 30 seconds) and transcript extension (72°C for 1 minute). The amplified product was size selected by electrophoresis in a 1% agarose gel, containing ethidium bromide for visualisation with a UV light source and using molecular weight markers (100 bp, Gibco) and further identified by sequencing.

The following parameters were changed in the previous protocol in order to optimise the PCR reaction for mRNA expression studies: 1) taq (10-25 units/ml), 2) dNTP (20-200 μM), Mg (0.5-2.5), annealing temperature (55-72°C), denaturation temperature and time (95-97°C for 60-15 seconds), cycle number (28-32) and primer concentration (0.1-0.5 μM). "Hot start" was also tested. The optimised PCR reaction mixture consisted of: 20 mM $(\text{NH}_4)_2\text{SO}_4$, 75 mM Tris-HCl pH 9.0 (at 25°C) 0.01% (w/v) Tween, 3 mM MgCl_2 (Advanced Biotechnologies Ltd.), 200 nM of each primer, 200 μM of each dNTPs, 8.3 units/ml Taq DNA polymerase (Advanced Biotechnologies Ltd.) and 2 μl of cDNA template and 30 μl of mineral oil in a final volume of 30 μl . The buffer, MgCl_2 and H_2O were previously treated with UV light for 5 minutes. Mineral oil (30 μl) was overlaid and the reaction began with an incubation at 96°C for 1 minute and 30 seconds, followed by 32 cycles of:

denaturation of double strand (94°C for 20 seconds), primer annealing (60°C for 30 seconds) and transcript extension (72°C for 1 minute).

Three sets of primers (Table 2.2) were assessed for suitability as housekeeping genes to monitor RNA integrity and the RT reaction, within the ovine species. The rat β -actin set was available in the laboratory from previous studies in the mouse species. The sequences of the human GAPDH set, were obtained from the literature (Baier et al., 1993). The ovine β -actin set (Oligo Express Ltd.) was chosen as the most appropriate housekeeping gene for mRNA studies. The ovine primers were designed from partial codings of an mRNA sequence found in the NCBI Gene Bank (accession number U08283) published by Bacich et al., (1994). When compared to the complete codings of the rat sequence, this fragment corresponded to the region encoding for aminoacids 300 to 354 (Nudel, 1983). General criteria in the design of primers were followed (described earlier in section 2.2.4ii), including the existence of an intron (124 bp) between the primers at least in the rat. The expected size for the RT-PCR products was 162 bp and approximately 286 bp in the event of genomic DNA contamination.

Primer set	Nucleotides	Sequence (5' to 3')	Size of product
ov actin	— —	TGG CAC CAC CAT GTA CCCT CAT CTG CTG GAA GGT GGAC	162 bp
rat actin		TTG GCC TTA GGG TTC AGG GGG G GGC CGC CCT AGG CAC CA	245 bp
hu GAPDH	4-22 bp 286-303	GTG AAG GTC GGA GTC AAC G GGT GAA GAC GCC AGT GGA CTC	300 bp

Table 2.2:primers used to amplify the housekeeping genes

iii) Semiquantitative RT-PCR of EP₃ mRNA

Total RNA was extracted as described before (in section 2.2.2) from tissues in which the EP₃R was known to have a function (kidney, liver and uterus) and from the cervix at different stages (oestrus, early luteal and prepartum). The RNA to be used (6 µl) was pre-treated with RNase-free DNase I as described before in section 2.2.3.

First-strand cDNA was synthesised from this pre-treated RNA as described before (section 2.2.4i). A semiquantitative PCR method was used to quantify mRNA expression encoding for the ovine EP₃R. The method, originally developed for the quantification of steroidogenic sequences by others (O'Shaughnessy and Murphy, 1993), was based on previous studies (Murphy et al., 1990).

The semiquantitative RT-PCR method is designed to measure the ratio of gene expression between EP₃R and the housekeeping gene β -actin, the later is used as an internal standard. β -actin is a valid RNA standard, because it is constitutively expressed in most cells and its levels of expression do not vary significantly with physiological changes (Gause and Adamovicz, 1994). The measurements are carried out in the exponential phase of the PCR, when the efficiency of the PCR is maximal (usually between 15-30 cycles, depending on the amount of starting material) and therefore the initial amount of target sequence is doubled on each amplification round. Doubling would occur in a PCR working with a 100% efficiency, but in practice only a 70-80% efficiency can be expected (Murphy et al., 1990; Ferre et al., 1992; Gause and Adamovic, 1994).

The exponential phase was determined for the EP₃R and β -actin cDNA amplifications. This was between 16 to 21 cycles for ovine β -actin and between 20-28 for the EP₃R. Eight PCR reactions were prepared for each sample, four identical PCR reactions for the EP₃R and another four for

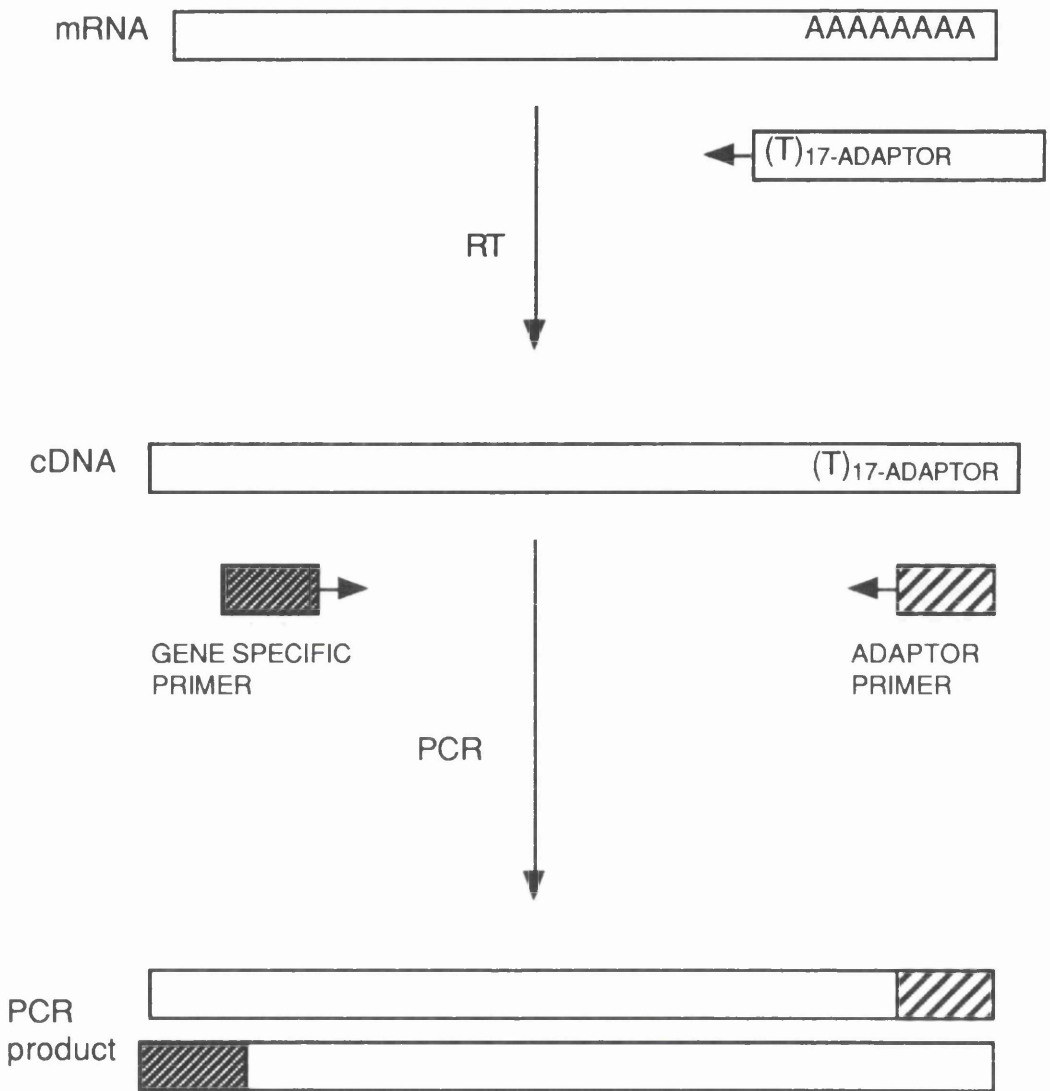
the house keeping gene, during different cycle numbers over the exponential phase. To measure product formation [³²P] α-dATP (1.5 μCi, specific activity of 3000Ci/nmol, ICN Flow) was added to each PCR reaction tube, prepared as described before (in section 2.2.4ii). PCR products were resolved in a 1% agarose gels, dried and autoradiographed. Bands were cut in a UV transilluminator using the ethidium bromide staining and the autoradiographs. Other areas, of the size of the bands, were also cut from the gel for determination of background levels. Radioactive incorporation was counted by liquid scintillation.

2.2.5 Isolation and characterisation of cDNA encoding the ovine EP₃ receptor isoforms using 3' end RACE (RAPID AMPLIFICATION OF cDNA ENDS).

RACE is another of the multiple applications of the PCR. It can be used for the isolation of novel sequences instead of the more tedious preparation and screening of libraries. The main strategy of this method is shown in a simplified diagram of the original diagram from Frohman et al., 1988 (Fig. 2.2.). Conventional PCR amplifies cDNA between two stretches of known sequences (primers), separated typically by 300-400 bp. But RACE, aims at a harder task; the amplification of cDNA knowing only a single stretch of the sequence to obtain the whole sequence, directing the extension of the primer to either the 5' end or the 3'end (RACE). In this study, because the EP₃R mRNA experiences alternative splicing at the C terminal, the use of 3'end RACE was particularly relevant to study the different isoforms produced. Other names for the technique includes "one sided PCR" or "anchored PCR", because the PCR is performed between a single gene specific primer and a adapter primer, the latter have to be anchored to the first strand during the RT. The adapter primer consists of a known sequence and can be used universally to isolate any novel sequence.

Figure 2. 2.

3' End rapid amplification of cDNA ends (RACE) diagram. Synthesis of 1st strand cDNA is performed with the (T)₁₇ adapter primer and reverse transcriptase. During the RT the (T)₁₇ anneals to the polyA tail and the adapter sequence gets incorporated into the new cDNA. A PCR is then carried out using a gene specific primer and the adaptor primer.



In short (see Fig 2.2), mRNA was reverse transcribed into single strand cDNA from total RNA with Moloney Murine Leukaemia Virus RNase H⁻ Reverse Transcriptase (SuperScript, Gibco) and the reaction was primed by an hybrid primer (oligo-dT17 -adapter), that consists of an oligo-dT of 17 residues linked to a unique sequence of another oligonucleotide. The strategy depends on the priming of the RT reaction by annealing of the oligo-dT part to the poly-(A) tail that most mRNAs have and also the incorporation of a known sequence (adapter part of the RT primer) at the 5'end of each cDNA created. The PCR is carried out with a sense primer which is gene specific for the target sequence and the adapter primer which is non-specific and is the antisense primer (Frohman, 1988 & 1990). In the first round of amplification the extension of the gene specific primer, working on the first strand of cDNA should create templates for the adapter primer. From this point the PCR between the gene specific and the adapter primer should work like any ordinary PCR (Figure 2.2). Three sets of three primers each were used in these experiments (Table 2. 1 and Figure 2.3).

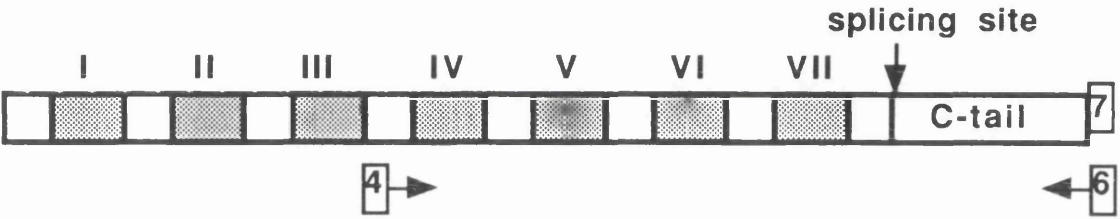
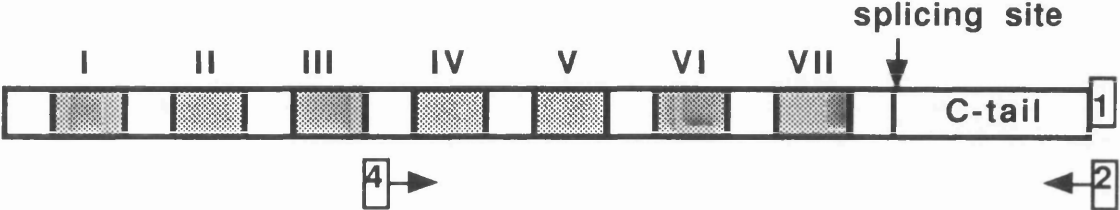
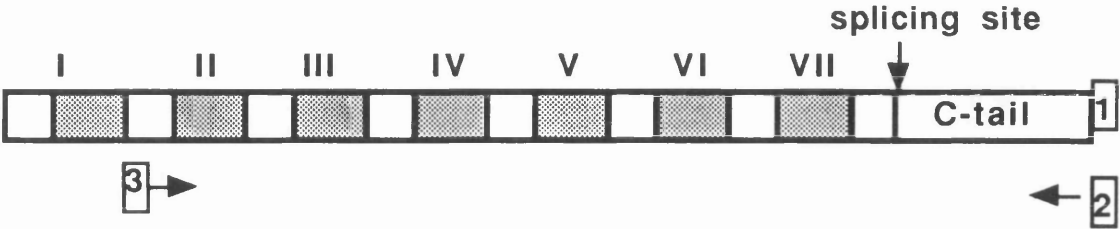
In the first set of primers, the sense primer or gene specific primer (P3) was designed from the bovine sequence of the EP₃ receptor (Namba et al., 1993) following similar criteria as explained in section 2.2.4ii. But at this early stage only bovine, mouse and rat sequences were available. The selected primer was checked for absence of homology with other sequences of prostanoid receptors published, but other mammalian species stored in the EMBL Gene Bank were not available to us at that time. The adapter primer (P2) is used in this method as an antisense primer (Frohman, 1988). The RT (1) and sense primer (P3) were obtained from Oligo Express Ltd. Positions and minimal sizes of expected PCR products are shown in Table 2.1 (page 107). Expected sizes are only orientative and were calculated from the longer and shorter bovine isoforms, and variable

length of the alternative transcripts of the EP₃R can be expected in different species. In addition, the size of the expected products is variable because the dT region before the adapter can have variable lengths, depending on where the RT primer is incorporated at the poly- (A) tail of the mRNA (Taniguchi et al., 1993).

The sense primer (P4) previously used in ordinary RT-PCR with an internal antisense primer (set 2, described earlier in section 2.2.4) was used with two sets of RACE primers (either P1 and P2 or P6 and P7). The adapter (external antisense, P6) primer and the RT primer (P7) sequences were those of Frohman's original protocol (Frohman et al., 1988), they were obtained from British Biotechnology Products. Positions and minimal sizes of expected PCR products are indicated in table 2.1 (page 107).

Figure 2.3

Position of the primers used in RACE experiments, relative to the bovine cDNA sequence. Two sets of RACE primers were used, either 1 and 2 or those of Frohman's original protocol (6 and 7). RT primers are 1 and 7. Adaptor primers are 2 and 6 and gene specific primers are 3 and 4. Because of the alternative splicing, products of different sizes were expected.



i) cDNA synthesis by Reverse Transcription (RT) in the RACE method

Briefly, RNA template (1,5 µg/ µl, approximately 0.03-1 µg) was added to a reaction mixture (20 µl) consisting of: 50 mM KCl, 10 mM Tris-HCl pH 8.3 (at 25°C), 4 mM MgCl₂ (Advanced Biotechnologies Ltd.), 1 mM dithiothreitol, 0.025 µg/µl of primer (oligo-dT + adapter), 100 mM of each dNTP, 0.6 units/µl RNase block (rRNasin, Promega) and 3 units/µl M-MLTV reverse transcriptase (SuperScript RNase H⁻, Gibco). The buffer (KCl, Tris-HCl and MgCl₂), H₂O and dithiothreitol were previously treated with UV light for 5 minutes. The reaction was incubated at room temperature for 5 minutes then at 37°C for 1 hour. The cDNA synthesised was stored at -20°C until used as template for the PCR reaction.

ii) RACE PCR: between gene specific and adapter primers

The PCR reaction mixture consisted of: 20 mM (NH₄)₂ SO₄, 75 mM Tris-HCl pH 9.0 (at 25°C) 0.01% (w/v) Tween, 2.5 mM MgCl₂ (Advanced Biotechnologies Ltd.), 400 nM of each PCR primer, 200 µM of each dNTPs, 8.3 units/ml Taq DNA polymerase (Advanced Biotechnologies Ltd.) and 3 µl of cDNA template and in a final volume of 30 µl. The buffer, MgCl₂ and H₂O were previously treated with UV light for 5 minutes. Mineral oil (30 µl) was overlaid and the reaction was begun with an incubation at 95°C for 3 minutes, followed by 32 cycles of: denaturation of double strand (94°C for 20 seconds), primer annealing (50-60°C for 30 seconds) and transcript extension (72°C for 1 minute and 30 seconds or 2 minutes).

The amplified product was electrophoresed in a 1% agarose gel, containing ethidium bromide for visualisation with a UV light source and

using molecular weight markers (plasmid pUC or 100 bp ladder). Further identification of the products obtained with the 1st set of primers was done by sequencing and of those obtained with the 2nd set by Southern blot analysis, using an internal fragment amplified by PCR (second set of primers in Table 2.1) as the probe.

iii) Control experiments with mice

The feasibility and the conditions of RACE were studied in a known system (O'Shaughnessy and Murphy, 1993; O'Shaughnessy et al., 1994) before starting the experiments with the ovine EP₃ receptor. The mRNA expression of gonadal proteins of different abundance (P450_{SCC}, ABP, P45017 α) and a housekeeping gene (β -actin) in the mouse gonad were studied, using primers that work for ordinary RT-PCR. The 1st set of RACE primers were tested in the mouse testis and in the mouse ovary. The RT of RACE was compared with other methods of Reverse Transcription (random hexamers and oligo-dT) and the 3' end RACE PCR was compared with ordinary PCR (using the 2 specific primers for the above mentioned genes). Two most likely limiting factors of RACE were investigated: maximal size of the product and minimal abundance of the target sequence.

2.2.6 Northern blot analysis

Northern blot analysis was carried out following the method of Sambrook et al., (1989).

i) Gel electrophoresis of RNA

A stock solution of sterile (by autoclaving) 10x MEA buffer (pH 7.0, adjusted with NaOH) was prepared consisting on 0.4 M MOPS, 0.01 M EDTA and 0.17 M sodium acetate. Samples of total RNA (5-30 μ g per lane) from each

tissue were denatured in 3 vols. of sample buffer (10 ml of 10x MEA, 35 ml of 37% formaldehyde and 100 ml of formamide) and incubated for 15 minutes at 65°C. A denaturing 1% agarose gel was prepared in 1x MEA buffer and made 6.4 % with respect to formaldehyde. Prior to loading samples, 0.2 volumes of 6x loading buffer (50% glycerol, 1 mM EDTA, 0.25% bromophenol blue and 0.25% xylene cyanol FF) and 2 µl of ethidium bromide (1 mg/ml in DEPC treated water) were added. The denaturing gel was placed in the electrophoresis tank with 1x MEA buffer and pre-run at 5 volt./cm for 5 minutes. The RNA samples were then loaded and electrophoresed at 1.5-2 volts/cm for 24 hours (until the bromophenol blue had migrated 8 cms from the wells) in 1x MEA buffer.

ii) RNA transfer

The gels were photographed with a ruler under UV light for size determination using the ribosomal bands (18S and 28S). Gels were then blotted to Biotrans (+)TM nylon membranes of pore size 0.45 µm (ICN) in 20x SSC by capillary elution. The RNA was fixed to the membranes by cross-linking with UV light, using the “UV Stratalinker 2400” at the setting for optimal cross linking. Membranes were stored wet (2x SSC) and wrapped in Whatman paper in the fridge until hybridisation.

iii) Radiolabelled probe making

³²P-labelling of cDNA probes was carried out using a random primer labelling kit (Prime-it TM II, Stratagene). A 355 bp cDNA obtained by PCR (see section 2.2.4 RT-PCR) was labelled with 50 µCi [α ³²P]-dATP (ICN) in a 50 µl reaction, according to manufacturers' instructions. Template was prepared as for sequencing (see later section 2.2.7) and the amount of DNA estimated by electrophoresis in a 1% agarose gel stained with

ethidium bromide (10 ng of DNA give a clear band). Briefly, 25 ng (1-23 μ l) of template were mixed with 10 μ l of random 9-mer primer (27 OD units/ml) to a final 32 μ l reaction and boiled at 96°C for 8 minutes. After a brief centrifugation the following reagents were added on ice and mixed by pipetting: 10 μ l of 5x dATP primer buffer (buffering solution containing 0.1 mM of each dNTP), 5 μ l of [α 32 P]-dATP (3000 Ci/mmol) (ICN) and 1 μ l of Exo(-) Klenow (5 units/ μ l). The reaction was incubated at 37°C for 10 minutes and stopped by adding 2 μ l of stop mix (100 μ l 0.5 EDTA, pH 8.0).

The labelled cDNA was purified and the unincorporated [α 32 P]-dATP removed by chromatography on a 1 ml column (Sephadex G50 DNA grade, NICK TM columns, Pharmacia) and stored on ice until used.

iv) Hybridisation

The membranes were prehybridised in 10 mls of buffer (6x SSC, 50% formamide, 5x Denhardt's solution 100 μ g/ml of herring sperm previously boiled) for 2 hours at 42°C in Hybaid bottles in a Hybaid oven. The cDNA probe was denatured by heating it at 95°C for 5 minutes and added to the prehybridisation buffer. The membranes were incubated with this solution, overnight at 42°C.

v) washes

The membranes were washed with 50 mls of 2x SSC/0.1 SDS at 42°C for 15 minutes, followed by the more stringent 50 mls of 0.2 x SSC/0.1 SDS at the same temperature and a final wash of 0.1x SSC/0.1 SDS. To remove excessive background, three more washes of 20 minutes each with the solution of 0.1 (w/v) SDS at room temperature were done.

vi) stripping of blots

A solution of 0.1 % CDC (w/v) at 95°C was poured over the blot for 5-10 minutes (up to 30 minutes if necessary).

vii) autoradiography

The blot was covered with cling film and placed against a sensitive X-ray film (Hyperfilm-MP, Amersham Int plc., CEA AB, Sweden) in a cassette (Amersham) with an intensifying screen. The film was exposed at -70°C for 36 hours and was processed in an automatic X-ray processor.

2.2.7 Cycle sequencing

The sequences of the isolated cDNA fragments by 3'end RACE (1st set of primers) and by RT-PCR were determined in both directions by cycle sequencing using a kit (Cyclist TM Exo-Pfu DNA sequencing kit, Stratagene). This method is based on a PCR variation (linear amplification) and Sanger's dideoxy chain termination enzymatic method for sequencing modified for single strand DNA. Electrophoresis was carried out in a 0.4 mm thick polyacrilamide gel.

i) Template preparation

The PCR products to be sequenced were excised from the agarose gel and the DNA was recovered by electrolution and precipitation. Dialysis membranes were prepared according to Sambrook et al., (1989) and filled with 500 µl of 0.5M TAE buffer. The excised band was inserted and the membranes closed hermetically. The DNA was allowed to migrate out of the agarose and into the dialysis membranes in an electrophoresis tank containing 0.5 M TAE buffer at 80 mA for 30 minutes, and for 5 minutes with

reverse polarity. The DNA recovered in the TAE buffer was then precipitated in the presence of 1/10 vol. of sodium acetate (pH 5.2) and 2 volumes of absolute cold ethanol (-20°C). The DNA pellet was resuspended in 5 µl of TE buffer (pH 8), 1 µl was run in an agarose gel to evaluate the amount of DNA present and the rest was stored at -20°C. To obtain a large quantity of DNA, one or two reamplifications were done, depending on the initial amount. To do this a dilution (1/100) of the template was reamplified by PCR in a 100 µl reaction (protocol as the 1st PCR). 20 µl of the reaction were run in a 1% agarose gel to check that the reamplification was successful. The other 80 µl (future sequencing template) was purified by precipitation in the presence of sodium acetate (pH: 5.2) and ethanol, resuspended in 20 µl of TE buffer and run in 1% agarose gel. The sequencing template was recovered from the agarose gel by electrolution and precipitation, resuspended in 5 µl of TE buffer (pH: 8) and stored at -20°C until used for sequencing.

ii) Sequencing reactions

8 sequencing reactions, 4 each for the 2 primers and one each for the 4 dideoxynucleotide (ddNTPs), were prepared according to manufacturers' instructions of the kit (Cyclist™ Exo-Pfu DNA sequencing kit, Stratagene) for each template.

Each sequencing reaction (total volume 10 µl) consisted of:

1x sequencing buffer (20 mM Tris-HCl (pH 8.8), 10 mM KCl, 2 mM MgSO₄, 100 µM (NH₄)₂SO₄, 0.1 % Triton, 0.1 mg/ml bovine serum albumin (BSA), 2 µM dATP, 5 µM dCTP, 5 µM dGTP and 5 µM dTTP), 106 nM of template, 250 µCi/ml of [α³⁵S] dATP, 62.5 U/ml of Exo-Pfu DNA polymerase, 0.1 µl dimethyl sulphoxide (DMSO), 100 nM of one of the two primers, 7.5 mM of one of the 4 ddNTPs and 15 µl of mineral oil.

The reactions were begun with an incubation at 95°C for 3 minutes, followed by 30 cycles of: denaturation of double strand (94-95°C for 20 seconds), primer annealing (60°C with RACE primers P45-P8 and 55°C with PCR primers P58-P61) for 30 seconds and transcript extension (72°C for 1 minute). The reaction were terminated with 5 µls of “stop solution”, consisting on 80% formamide, 50 mM Tris-HCl (pH 8.3), 1 mM EDTA, 0.1 % bromophenol blue and 0.1% xylene cyanol. The reactions were stored frozen at -20°C until the moment of loading them in a sequencing gel.

iii) Electrophoresis

Making the sequencing gel

The following recipe was used for a 75 ml gel: 15 ml of 40% acrylamide (38% acrylamide and 2% N’N’bis acrylamide), 15 mls of TBE buffer, 18 ml of double deionized water (ddH₂O), 36 grams of urea, 0.75 ml of 10% ammonium persulphate and 22.5 µl of N,N,N’,N’ tetramethylethylenediamine (TEMED). The 40% acrylamide, TBE buffer, ddH₂O and urea were dissolved in the microwave by heating to a maximum of 37°C and allowed to cool to room temperature. When ready to pour the gel, the polymerisation agents were added: freshly prepared 10% ammonium and TEMED. Holding the casting apparatus at an angle of 45° the solution was quickly poured down one corner at a steady rate, using a 50 ml syringe. The gel was returned to an almost horizontal position (5°) and an even levelled border was created by inserting a reversed comb up to 3-5 mm deep. The gel was allowed to polymerise overnight at room temperature, keeping the top moist and being covered with Saran wrap.

Running the gel

Before loading the gel was pre-run for 1 or 2 hours with 0.5 TBE buffer at 1850 volts to heat the gel and the buffer to its running temperature 50°C. The sequencing reactions were heated to 95°C for 3 minutes to denature and concentrate the samples and 2 µl of sample were quickly loaded in the order GATC GATC for each primer. The samples of the first loading were run for 6 hours and then two more loadings were done separated 2 and 5 hours from the first loading.

Fixing and drying the gel

The gel was fixed with 3 litres of a solution (v/v) 10% methanol/ 10% acetic acid for 5 minutes and left to dry over filter paper in a vacuum gel-dryer at 80°C for 3 hours.

iv) autoradiography

The gel (supported by the filter paper) was placed in a cassette with film (Kodak). Film was exposed for 72 hours at -70°C and developed using an X-ray processor.

2.2.8 Southern blots

Southern blots were carried out according to O'Shaughnessy et al. (1993).

i) DNA transfer

The RT-PCR electrophoresis gels were photographed with a ruler under UV light for size determination using DNA molecular markers (100 bp, Gibco). Gels were then blotted to Biotrans (+)TM nylon membranes (ICN) in 20x SSC by capillary elution. The DNA was fixed to the membranes by baking at 80°C for 15 minutes. Membranes were stored wet (2x SSC) and wrapped in Whatman paper in the refrigerator until hybridisation.

ii) Hybridisation

The membranes were prehybridised in 10 mls of buffer (5x SSC, 0.1 % SDS, 5x Denhardt's solution, 100 µg/ml of herring sperm) for 1 hour at 63°C in Hybaid bottles in a Hybaid oven. The cDNA probe was denatured by heating it at 98°C for 5 minutes and added to the prehybridisation buffer. The membranes were incubated with this solution, overnight at 50°C.

³²P-labelling of cDNA probes was carried out using a random primer labelling kit (Prime-it TM II, Stratagene) as described earlier for northern blot analysis (2.2.6).

iii) washes

The membranes were washed with 50 mls of 2x SSC/0.1 SDS at 55°C for 15 minutes, followed by the more stringent 50 mls of 1 x SSC at the same temperature.

iv) autoradiography

This was done as for the northern blots (2.2.6) exposing the film for 1, 2 and 24 hours.

CHAPTER 3

THE USE OF PROSTAGLANDIN E₂ (PGE₂) TO INDUCE CERVICAL RIPENING IN EARLY LUTEAL PHASE SHEEP

3.1 INTRODUCTION

3.1.1 Use of PGE₂ for the induction of cervical ripening during the cycle and pregnancy

The usefulness of prostaglandins for the induction of labour at term or for the therapeutic termination of pregnancy in early gestation in humans were first documented in the late 1960s and early 1970s. Initially, the rationale of using these drugs was the discovery in the 1930s, that prostaglandins could induce uterine contractions. Later, it was discovered that doses insufficient to induce uterine contractions, induced cervical softening (reviewed by Keirse, 1990; Robinson et al., 1991). In the 1970s and 1980s, the ovine cervix was used as an experimental model to investigate further the effect of prostaglandins in the cervix (Fitzpatrick, 1977; Fitzpatrick and Liggins, 1980). Several experiments demonstrated that prostaglandins softened the cervix by a direct effect rather than the induction of uterine contractions. Initially PGF_{2α} was used for the induction of labour in humans, but later the uterotonic superiority of PGE₂ became evident. Because of the previous clinical experience with the E series in the uterus, in most countries PGE₂ has also been used more than PGF_{2α} for cervical ripening, except in Australia where PGE₂ was not licensed until recently (MacLennan, 1990). There are only two studies comparing both PGs, the results of these studies may not be absolutely convincing (reviewed by Keirse et al., 1990) but support the superiority of the PGE₂ for the induction of cervical ripening, a view held by most clinicians (Calder, 1990).

Clinical experience has shown that the intravaginal or endocervical routes are preferred in comparison with other local routes (intraamniotic) or the general routes (oral and intravenous). Lower doses can be used with the local routes, minimising side effects and cost (Calder and Greer, 1992;

Calder, 1994). The naturally occurring prostaglandins are rapidly catabolized in a single passage through the lungs, but synthetic analogues of PGE₂ and PGF_{2α} have been developed with the intention of prolonging their half life and increasing their potency. These molecules have also been designed to have a more selective action on the reproductive tract, reducing their side effects (Hess et al., 1979; Coleman et al., 1990).

Prostaglandin E₂ (PGE₂) analogues are used to induce preoperative cervical ripening in humans, before termination of pregnancy at the 2nd and 3rd trimesters (Bygdeman, 1992). Another very common indication for PGE₂ has been the ripening of the unfavourable cervix at term, in this case the natural compound is usually preferred. The E series has been widely used clinically during pregnancy for these purposes, but its use during the cycle is far less documented and results at this stage are not very clear (reviewed by Ulbjerg, 1989; Calder and Greer, 1992). Early reports suggested that PGs have a similar effect at both reproductive stages (Rabe, 1985). More recent works have shown that cervical ripening with prostaglandins can be induced in non-pregnant women; but results are either poorer than during pregnancy or undifferent when compared with placebo (Sulprostone: Lueken and Lindermann, 1979; Ulbrich and Bartels, 1979; Gemeprost: Elder and Lewis, 1991).

In sheep it is clear that PGE₂ is effective inducing cervical ripening during late pregnancy (120-140 day gestational age) and it is less effective in animals with high levels of progesterone (Fitzpatrick, 1977; Stys et al., 1981; Owiny and Fitzpatrick, 1990). Recently there has been an interest in the use of this drug during the superovulated luteal phase (day 6 after oestrus) in order to develop a transcervical embryo transfer technique but the results have been variable. Some authors reported very successful embryo collection in South Africa (Barry et al., 1990) and others have reported variable results in Scotland (Audicana, 1992; Mylne et al., 1992). Partial

ripening and incomplete catheterization in anoestrous sheep has been reported (Rickords and White, 1988).

3.1.2 The microscopic changes of cervical ripening induced by PGE₂ or other ripening drugs

The mechanism of action of PGE₂ in the induction of cervical ripening is not fully understood. During physiological ripening, microscopic changes have been reported in sheep (Parry and Ellwood, 1981; Aughey et al., 1981; Fosang et al., 1984; Owiny et al., 1987), women (Danforth, 1960; Junqueira et al., 1980; Manabe and Yoshida, 1990; Knudsen et al., 1991), and other species. The changes consisted of separation of collagen fibres and is associated in most reports with the presence of inflammatory cells, these changes have been explained in detail in chapter I.

Similar studies have shown that the mechanism of cervical ripening when induced by PGE₂ and its analogues resemble those of physiological ripening in sheep and other mammals (sheep: Owiny et al., 1987; women: Uldbjerg et al., 1981; Rath et al., 1987; monkey: Shimizu et al., 1994 and the rabbit: Rayburn et al., 1991). Microscopic changes have also been reported following treatment with other prostaglandins, (PGF_{2α}: Theobald et al., 1982; MacLennan et al., 1985; Owiny et al., 1987; PGF_{2α} analogue: Manabe and Yoshida, 1990; PGE₁ analogues: Manabe and Yoshida, 1990; Greer et al., 1992). Similarly, these changes can be induced by other cervical ripening drugs in late pregnant women and experimental animals (relaxin: MacLennan et al., 1985; antiprogestagens: Hegele-Hartung et al., 1989; interleukins: Chwalisz et al., 1994). Changes in all the cases are similar to those reported during physiological cervical ripening.

There is biochemical evidence supporting that collagenolysis occurs in the periparturient period, during both physiological and pharmacological ripening induced by PGE₂. Collagenolysis may be responsible for the

increased spaces between collagen fibres observed in sections. Collagenase and elastase activities are increased following PGE₂ treatment, the effect is so rapid as to suggest a release from PML granules rather than new synthesis. The PML is known to store collagenase (MMP-8) and elastase, and therefore it is a potential source of these enzymes during PGE₂-induced cervical ripening (Rath et al., 1993; Doherty and Janusz, 1994). Others however have found that PGE₂ increases the synthesis of collagenase in the cervix *in vitro* (Goshowaki et al., 1988), which suggests the involvement of the macrophage or the fibroblast, which do not store but synthesise collagenase *de novo* (Woessner, 1994). Active PMLs, macrophages and fibroblasts have been observed in the cervix, following PGE₂ administration, but it has not been demonstrated which cell(s) and enzyme(s) cause ripening (Greer et al., 1992; Rayburn et al., 1994). Leukocyte elastase has been localised in PMLs following treatment with the PGE₂ analogue Gemeprost, but the authors did not find good correlation between the number of PMLs and the degree of cervical ripening (Greer et al., 1992). The author of this thesis is not aware of similar studies of fibroblast (MMP-1) or PML collagenase (MMP-8).

During the cycle, not only are there very few clinical studies of the effect of PGE₂ in the cervix, but also there is no information on the mechanism of action of PGE₂. Unlike during pregnancy, there are no morphological, mechanical or biochemical studies following the administration of PGE₂ or other drugs in sheep or other species. The aims of this study were therefore twofold:

1) The clinical assessment of intravaginal human preparations of PGE₂ for the purpose of facilitating passage of the cervix with a catheter, during the cycle and early pregnancy in sheep (experiment 1). This experiment was carried out to study the possibility of using PGE₂ to develop a non surgical (transcervical) embryo transfer technique.

2) The investigation of whether in the luteal phase, PGE₂ can induce microscopic changes similar to those observed during physiological ripening immediately before labour (experiment 2).

3.2 MATERIALS AND METHODS

3.2.1 Animals, oestrus induction, superovulation and matings

Adult cast Scottish Greyface were used for the present experiments. The sheep were bought from a dealer and accommodated in a covered straw-bedded house for the duration of the study.

Oestrus was synchronised in the ewes using a 12-day regimen of fluorogestone acetate intravaginal sponges (Veramix, Upjohn). Oestrus was expected in these sheep 48 hours after sponge removal. Rams were introduced 48 hours after sponge removal and kept for 2-3 days. Ewe/ram ratio was always within the recommended 10:1 or less.

Superovulation was induced by a single intramuscular (IM) injection of 1500 I. U. of equine chorionic gonadotrophin (eCG) (PMSG, Intervet) in some sheep and was administered on the day of sponge removal. Oestrus

was expected in these sheep 24 hours after sponge removal and eCG injection. Rams were introduced 24 hours after sponge removal and kept for 2-3 days.

3.2.2. Experiments

Experiment 1: Transcervical catheterization of sheep following treatment with intravaginal PGE₂ pessaries

15 superovulated and mated sheep were examined at day 18 after oestrus. Pregnancy status was established by measuring plasma progesterone concentrations on day 16 using an ELISA kit (Ovucheck, Cambridge Life Sciences) according to Eckersall and Harvey (1987). The animals were allocated into 4 groups described later. Sheep were restrained as for AI for evaluation of the cervix and administration of the PGE₂, the hind quarters of the ewes were lifted and placed over a rail. The effect of the treatment was evaluated by probing the cervix with a 0.5 cm diameter insemination pipette with an angled tip and divisions of 1 cm. The depth scored was recorded before and after the treatments and the control group were examined at corresponding times (+4.5 and 24 hours) following mock insertion of pessaries. The resulting *data* were analysed using a two way analysis of variance.

- 1) group C: control group that received no treatment (n=4)
- 2) group E: received 0.25 mg of oestradiol benzoate injected intramuscularly (n=3), the effect of this treatment was evaluated 20 hours later.
- 3) group P: PGE₂ group, received either one or two intravaginal pessaries containing 10 mg of Dinoprostone (Propess, Roussel Lab) (n=5). This was a new form of intravaginal administration, designed for slow release, at a steady rate of less than 1 mg per hour and therefore for approximately 12 hours *in vitro* (Rayburn et al., 1992). They were kept at -20°C until immediately before use. The effect of the 1st pessary was evaluated 4 hours

and 30 minutes and 24 hours later. A second pessary was inserted in 3 of these sheep 24 hours after the 1st and the effect was evaluated 4 hours later (+28 hrs) together with those sheep that had only received one pessary.

4) group PE: This group (n=3) was given one pessary of PGE₂ that was evaluated 4 hours and 30 minutes later, at this time the ewes were given an IM injection of 0.25 mg of oestradiol benzoate. The effect of oestradiol was evaluated 24 hours later, which was followed by the administration of a second pessary to be evaluated 4 hours later (+28 hrs) and the next day (+48 hrs).

Experiment 2: Microscopic study of the cervix following PGE₂ treatment

In two trials, two intravaginal preparations of PGE₂ (4 mg of a gel or 10 mg of a vaginal controlled release pessary) were administered on day 6 of the cycle to ordinary (T-, n=6) or superovulated animals (T+, n=6). The effects were evaluated after different exposures (24 hours and 1 week) in the ordinary luteal phase and (8, 16 and 24 hours) in the superovulated animals. Control groups were available at day 6 of the normal cycle (C-, n=2) and of the superovulated cycle (C+, n=3) (Table 3.1).

Sheep were sacrificed with an intravenous injection of 20% pentobarbitone sodium (Euthetal, RBM). In trial 1, before the euthanasia some sheep (n=3) were anaesthetised with 6% pentobarbitone sodium (Sagatal, RMB) and catheterization with a catheter of 2 mm diameter was attempted. In trial 2, this was omitted to be able to differentiate between microscopic changes due to PGE₂ or the catheterization.

Specimens were collected immediately postmortem from the 4 groups (see Table 3.1), at the cranial vagina, and at three areas of the cervix (external os, mid cervix and internal os) to be processed for light microscopy (LM), transmission (TEM) and scanning electron microscopy (SEM) (see chapter II).

Trial	Group	n=	day -1 (eCG)	day 6 (PGE₂)	sacrifice day
2	C -	2	---	---	day 7 (n=2)
1	C +	3	1500 IU	---	day 7 (n=3)
2	T -	6	---	gel (4 mg)	day 7 (n=3) day 12 (n=3)
1	T +	6	1500 IU	pessary (10 mg)	day 6 and * 8 hrs (n=2) 16 hrs (n=2) * 24 hrs (n=2)

Table 3.1: Treatment groups in trials 1 and 2. of Experiment 2 (Microscopy)

Trial 2: None of the sheep were superovulated (C- and T-). The control group (C-) received no further treatment and the treated group (T-) were given an intravaginal gel containing 4 mg of Dinoprostone (Prostin, Upjhon) on day 6 of the cycle (oestrus: day 0). Trial 1: All the sheep were superovulated (C+ and T+) with an IM injection of eCG on day -1 (day of sponge removal). The treated group (T+) received another preparation of PGE₂ on day 6 of the cycle, intravaginal pessaries containing 10 mg of Dinoprostone (Propess, Roussel Lab). Only in this trial sheep (*3/6) were attempted to be catheterized under general anaesthesia before sacrifice.

3.3 RESULTS

3.3.1 Catheterization of the cervix and softening

Complete catheterization was not possible in any of the ewes at day 18 with a catheter of 5 mm in diameter (experiment 1: Tables 3.2, 3.3 and 3.4). In experiment 2 (at day 6, under anaesthesia and with 2 mm catheter, catheterization was incomplete in the ewes (n=3) (trial 1), measurements were not recorded because the samples had to be collected fast for microscopy (results therefore are not shown). The consistency of the cervix was softer in PGE₂-treated sheep than in control sheep.

The cervix in the luteal phase of control sheep of the breed Scottish Greyface was 7-10 cm long (observed postmortem: Plate 3.1). The maximal depth of catheterization in control sheep *in vivo* was variable ranging from 0.1 to 3 cm, these differences were not significant statistically. Variation was observed when probing the cervix of the same ewe at different times and between ewes. Deeper catheterization (>3 cm), was observed with the following treatments: oestrogen after 20 hours (in 1/3 ewes) or a single pessary of PGE₂ after approximately 4 hours and 30' and after 24 hours (1/5 and 5/5 respectively). Only the treatment with PGE₂ after 24 hours was significant ($p \leq 0.001$). The maximal depth achieved was 5 cm in a single animal (1/6) after receiving a second pessary of PGE₂. Oestradiol priming did not improve the effect of a single or two pessaries of PGE₂, the statistical analysis showed that if any oestradiol had a negative effect on the PGE₂ treatment.

		Cervical depth (cm)		
ewe	pregnant	0 hr	4.30 hr	24 hr
C1	yes	3	0.1	—
C2	yes	3	3	1
C3	yes	0.1	2.5	1
C4	no	0.1	2.5	1

Table 3.2 Cervical depth measured at time intervals following mock pessary insertion in control group.

		Cervical depth (cm)			
ewe	pregnant	minus 28 hr	minus 24 hr	0 hr	plus 20 hr
E1	yes	—	3	—	0.5
E2	yes	3	3	1	2.5
E3	yes	0.1	3	1.5	4

Table 3.3 Cervical depth measured at time intervals following oestrogen treatment (Group E)

		cervical depth (cm)			2nd pessary	Cervical depth (cm)
ewe	pregnant	0 hr	plus 4.30 hr	plus 24 hr	inserted plus 24 hr	
P1	yes	0.1	0.1	4***	no	3 —
P2	yes	—	4	4	no	3 —
P3	no	0.1	1.5	4***	yes	5 —
P4	yes	0.1	0.5	4***	yes	1.5 —
P5	yes	0.1	3	4***	yes	2 —
PE1	yes	—	3	2	yes	1 —
PE2	no	3	p2	2	yes	3 2
PE3	yes	—	3	2	yes	2 *2

Table 3.4 Cervical depth in group P (PGE pessary) and group PE (PGE2 and oestradiol) at time intervals following treatment. p: pessary missing. ***: p< 0.001.

Plate 3.1

Complete catheterization of a reproductive tract post-mortem, reaching the right uterine horn (asterisk).

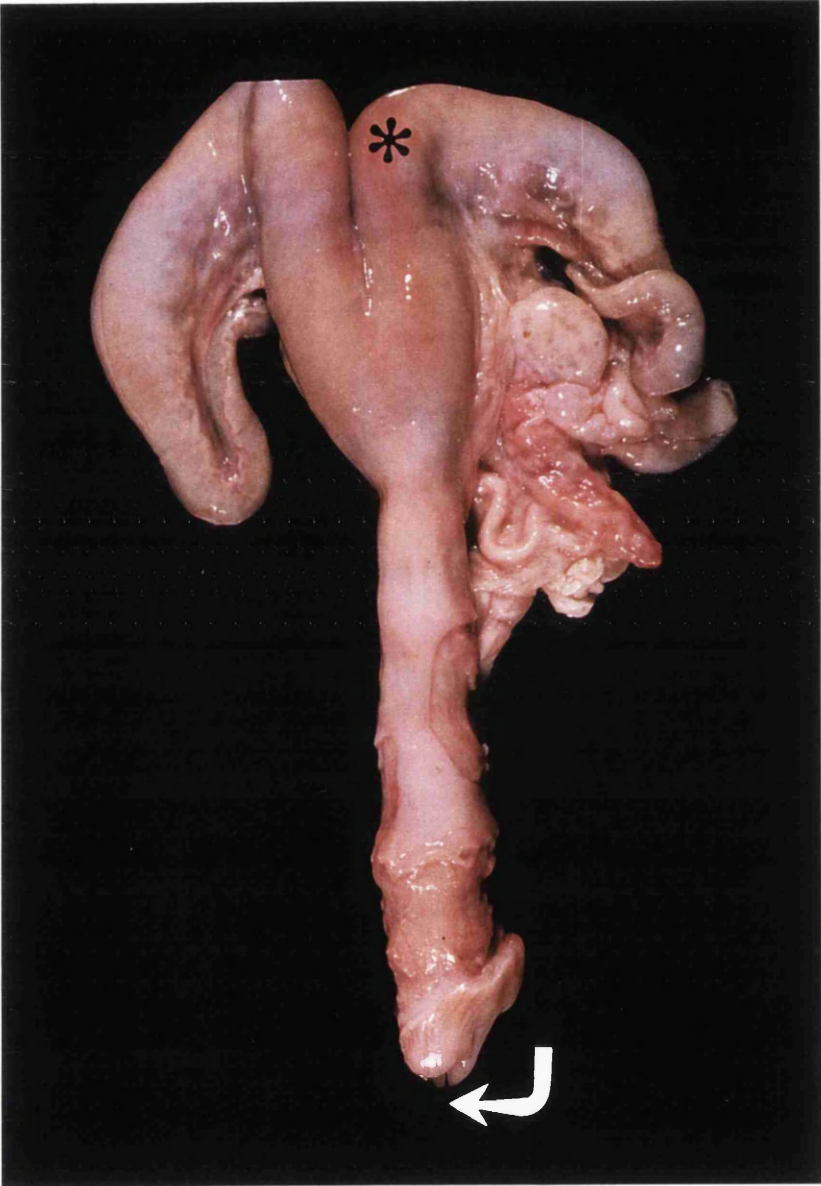


Plate 3.2

In contrast with the previous specimen, another tract shown here where one of the mucosal folds occludes the cervical canal completely.



3.3.2 Microscopic changes produced by PGE₂

Both groups of control sheep (C+ and C-) presented a similar epithelium and subepithelium. The vagina was lined by stratified squamous epithelium and the endocervix by a stratified squamo-columnar epithelium at the external os and a columnar epithelium in mid cervix and at the internal os. The mucosa of the vagina and endocervix consisted of dense connective tissue, the collagen fibres were compactly arranged and contained very few cells (fibrocytes and white blood cells). Eosinophils and PMLs were seen occasionally (1 per field) in the endocervix and were inactive (no degranulation). Extravascular RBCs were not observed in these groups (Plates 3.3-3.12).

An inflammatory response was observed in the two PGE₂ treated groups (T+ and T-), localised in the subepithelial connective tissue and epithelium of the cranial vagina (Plates 3.3.-3.6) and in the endocervix of the external os (3.7-3.13). This response was less obvious at the mid cervix (Plates 3.14-3.16) and the internal os (Plates 3.17-3.19). Light microscopy showed an infiltration of inflammatory cells in the subepithelial connective tissue and across the epithelium into the lumen. Blood vessels in the subepithelium were enlarged and contained abundant red blood cells (RBCs). There were areas of looser collagen, mainly in the endocervix but also in the fibromuscular wall of the cervix and there was an increase in the ground substance between the collagen fibres.

TEM showed that the inflammatory cells were PMLs, eosinophils, mast cells, platelets, macrophages and RBCs that were very abundant in blood vessels or in the connective tissue. The PMLs and the eosinophils were present in great numbers (>5 cells/field), both intravascular, leaving blood vessels and extravasated into the extracellular matrix. They showed haloes of collagenolysis around them, the collagen fibres were less densely arranged than in control animals. Transepithelial migration into the lumen

and degranulation was observed. Some eosinophils were phagocytosed by macrophages in the extracellular matrix. It was also common to find activated mast cells and platelets in the vicinity of PMLs and eosinophils.

SEM of these specimens showed the presence of RBCs and inflammatory cells, desquamation of epithelial cells and threads of mucus or fibrin (Plates 3.4 and 3.8). This inflammatory response was more intense in ewes that had longer exposure to PGE₂ and was localised in the cranial vagina and the most caudal parts of the cervix. The inflammatory response was reversible after one week in 2 out of 3 ewes. Microscopic results are summarised in Table 3.5.

Table 3.5: Morphological changes at the external os induced by PGE2. administered in the early luteal phase.
after the induction of oestrus (day 1) with(+) or without eCG (-)

GROUPS	DAY O eCG (1500 IU)	DAY 6 PGE form Dose	COLLAGEN FIBRES SEPARATION	INFLAMMATORY CELLS IN THE CONNECTIVE TISSUE							
				Ma	PC	RBC	Ly	PML	Eo	Plat.	MC
C- n = 2	-	-	1	0	2	0	2	0	0	0	0
C+ n = 3	+	-	1	0	2	0	2	1	1	0	0
T- n = 3	-	(prostin E , Upjohn) 4 mg GEL	3	1	2	3	2	3	3	2	2
T+ n = 6	+	(Propess, Roussel) 10 mg PESSARY	3	1	2	3	2	3	3	2	2

ABREVIATIONS: Ma = MACROPHAGES, PC = PLASMA CELLS, RBC = RED BLOOD CELLS, Ly = LYMPHOCYTES, PML= POLYMORPHONUCLEAR LEUKOCYTES, Eo = EOSINOPHILS, Plat. = PLATELETS, MC = MAST CELLS
KEY: SCORES = OCCURRENCE; 3 = MAXIMUM, 0 = NONE.

Plate 3.3

Light micrographs of the cranial vagina

Upper Control animal showing a stratified squamous epithelium. Magnification x20

Lower. Treated animal showing an inflammatory response in the epithelium (open arrows), in these areas white blood cells and red blood cells can be observed. The epithelial border (closed arrow) also presents infiltration of red blood cells and desquamation. Magnification x20

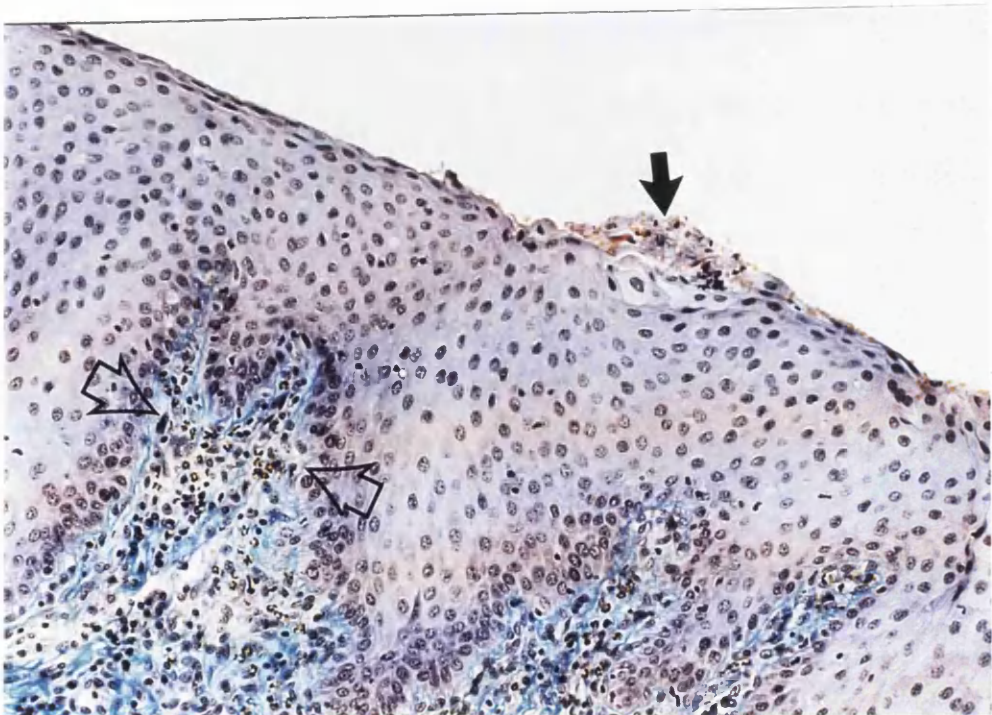
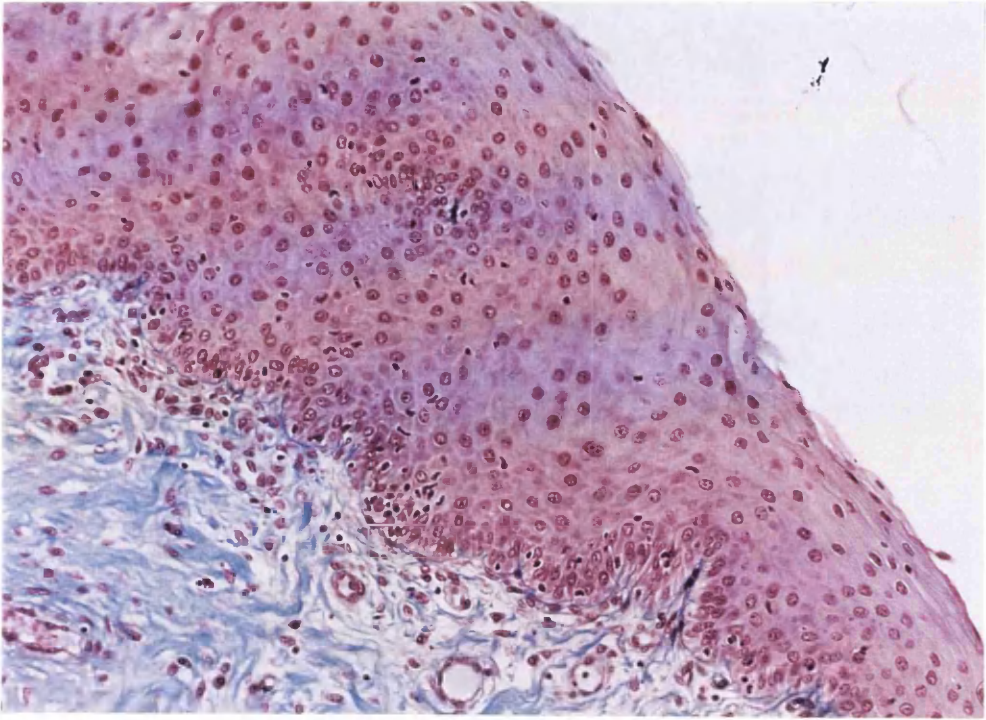


Plate 3.4

Scanning electron micrographs of the cranial vagina

Upper Control animal showing a stratified squamous epithelium. A few red blood cells is a normal finding.

Magnification x1440

Lower Treated animal showing an inflammatory response in the endocervix. Note desquamation of the epithelium, and presence of WBCs (arrow) with strands of mucus (Mu). x1440



Plate 3.5

Transmission electron micrographs of the subepithelium of the cranial vagina

Upper Control animal showing dense collagen (Co), a fibroblast (Fb) and absence of WBCs. Magnification x8K

Lower Treated animal showing a subepithelial inflammatory response with presence of polymorphonuclear leukocytes (PMLs) and eosinophil (Eo). Fibroblast (Fb) also present. Magnification x8K

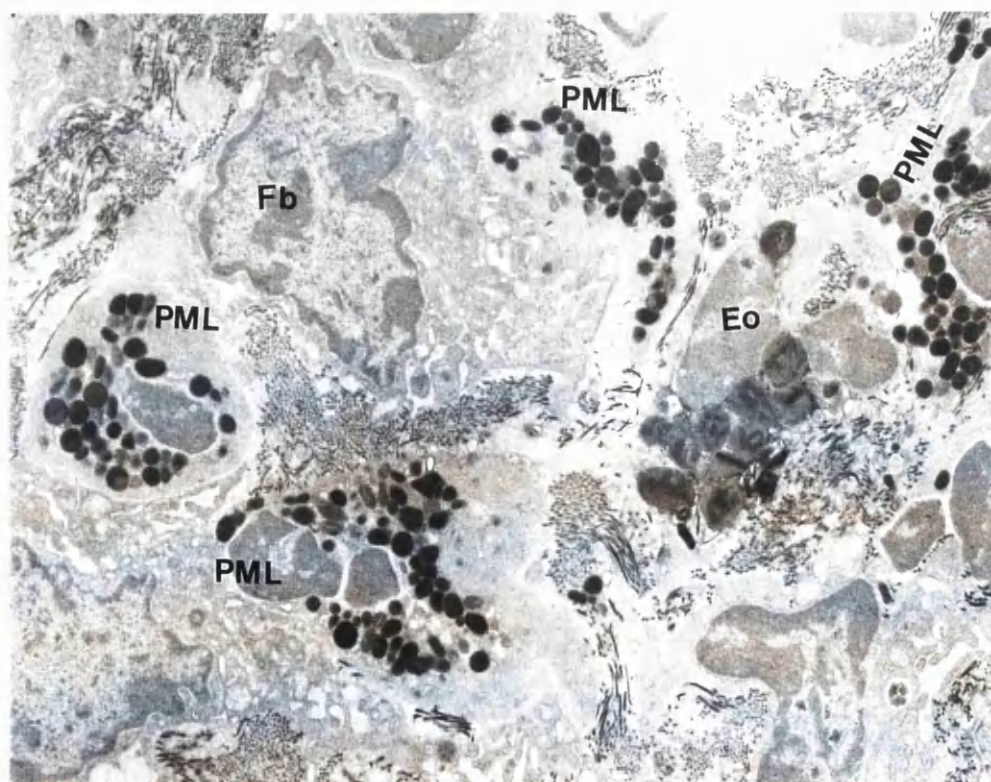
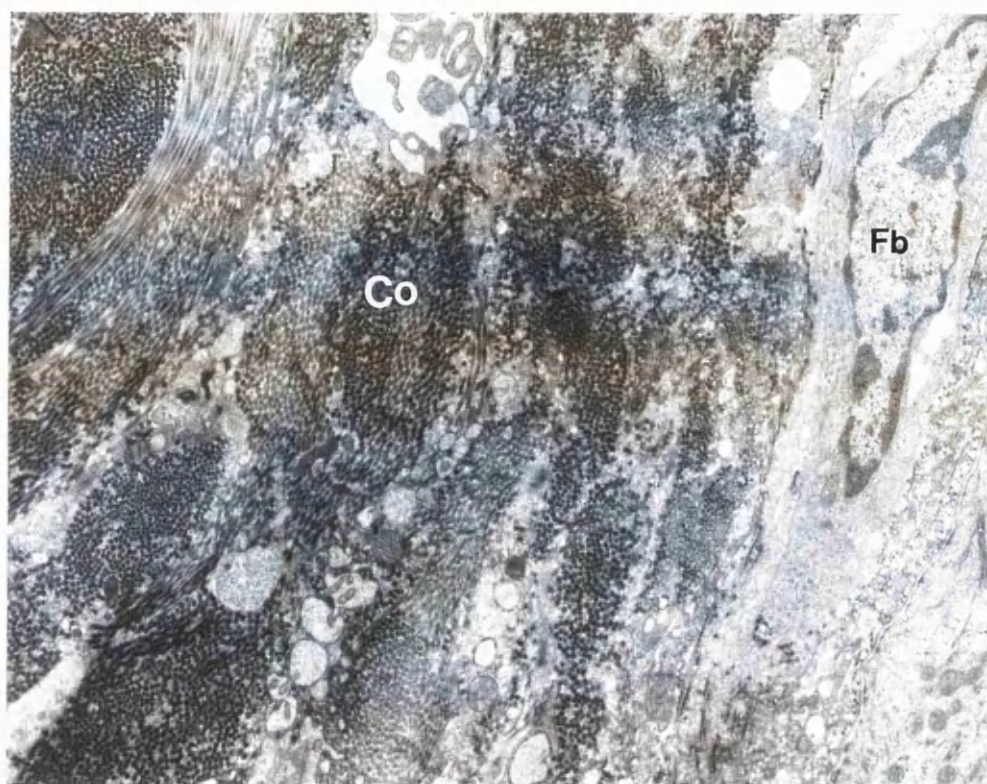


Plate 3.6

Transmission electron micrographs of the subepithelium of the cranial vagina

Upper Treated animal showing subepithelial free granules of PMLs (open arrow) under the epithelial border (closed arrow). x10K

Lower Treated animal showing a macrophage with phagocytosed eosinophilic granules. Note that it is not an eosinophil because it has a monocytic nucleus (Ma) and the granules are stored in vacuoles (open arrow). x16K

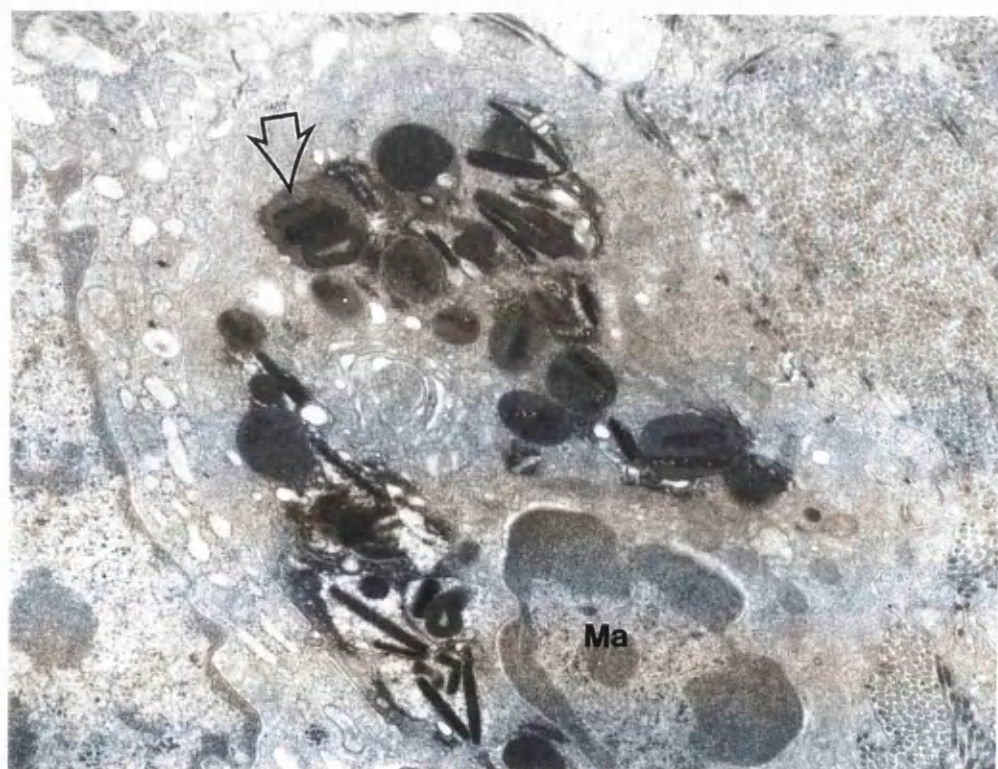
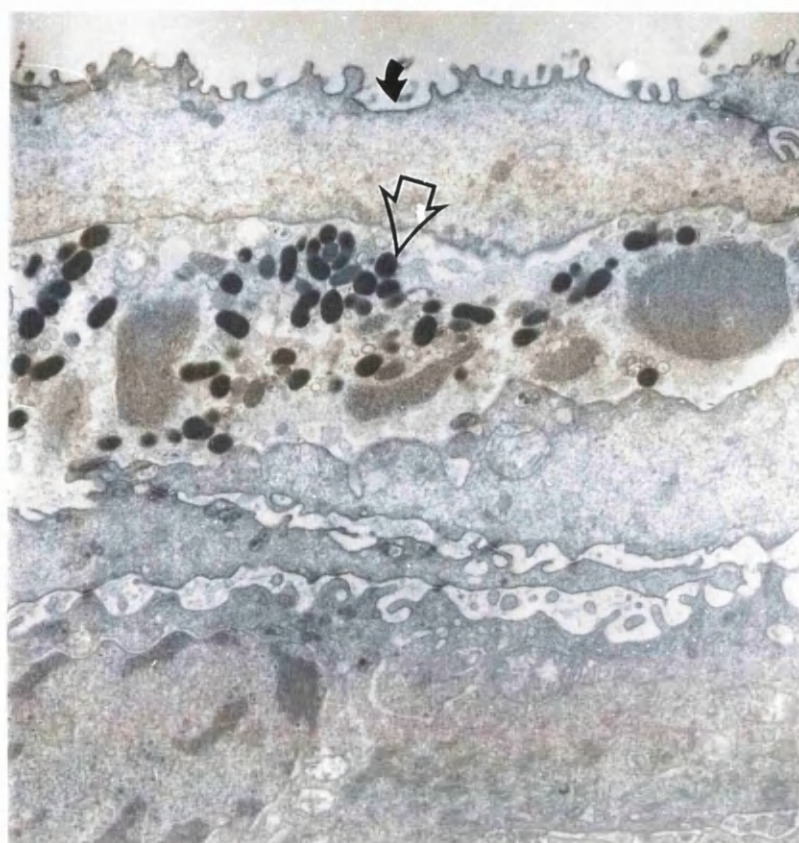


Plate 3.7

Light micrographs of the external os of the endocervix

Upper Control animal showing a transitional epithelium between columnar (c) and squamous (sq). Magnification x10

Lower Treated animal showing a subepithelial inflammatory response (open arrow), with the presence of white blood cells (WBC) and red blood cells (stained in yellow in the open arrow area). Note desquamation of the epithelium (closed arrow).

Magnification x10

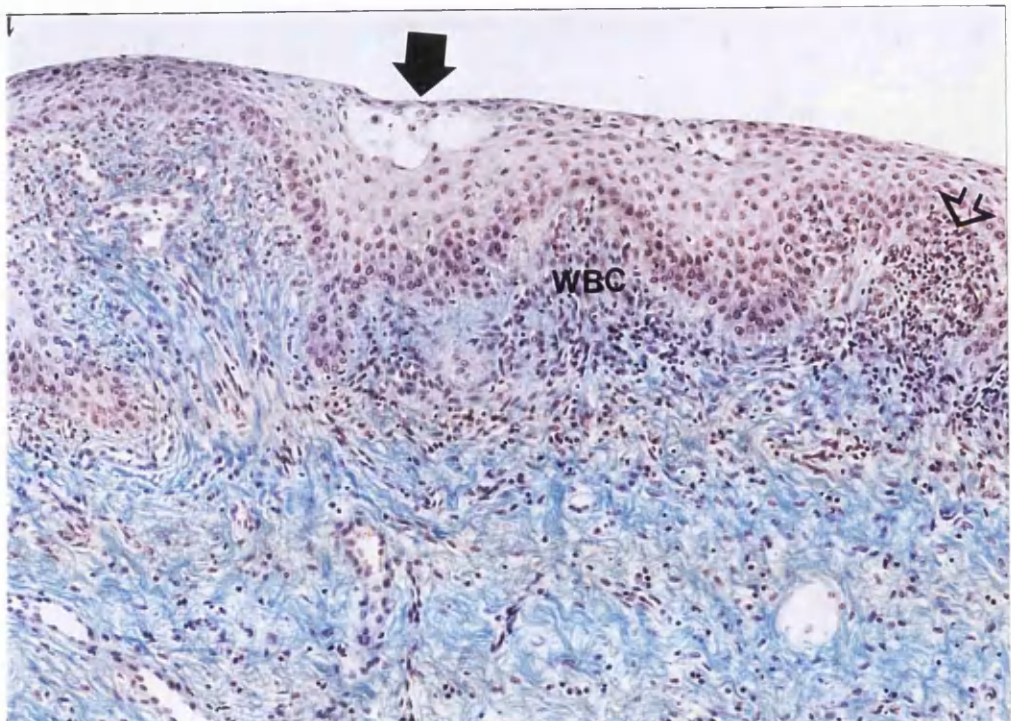
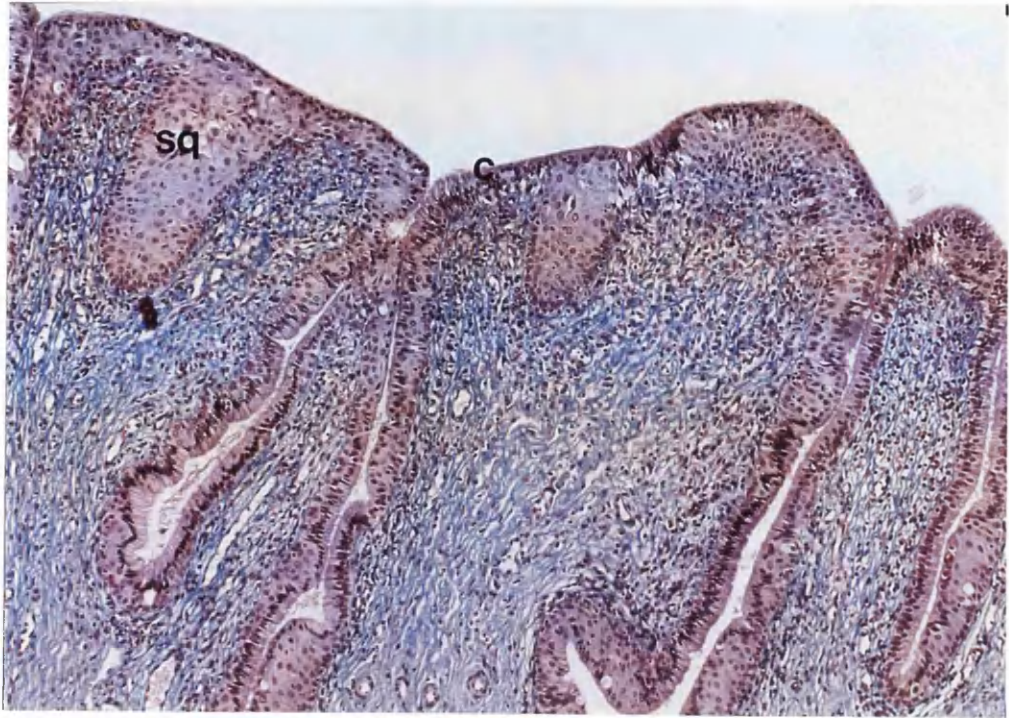


Plate 3.8

Scanning electron micrographs of the external os of the cervix

Upper Control animal showing a stratified squamous (sq) and columnar epithelium (c). Magnification x2813. note the presence of red blood cells (RBC)

Lower Treated animal showing an inflammatory response in the epithelium. Note the mucus and the presence of RBCs, squam (sq) and WBCs (arrows). Magnification x2813

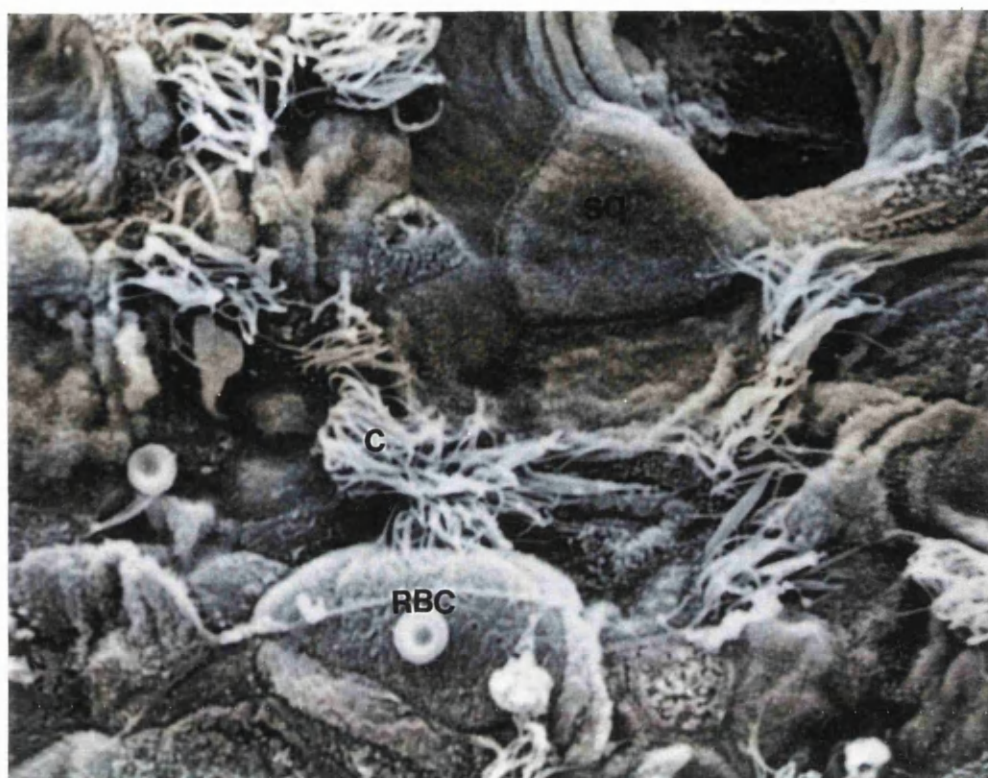


Plate 3.9

Transmission electron micrographs of the external os of the cervix

Upper Epithelial border (arrow) of a control animal showing secretory granules (SG) of mucus. Magnification x8K

Lower Epithelial border (arrow) of a treated animal showing eosinophilic granules (EoG). Magnification x8K

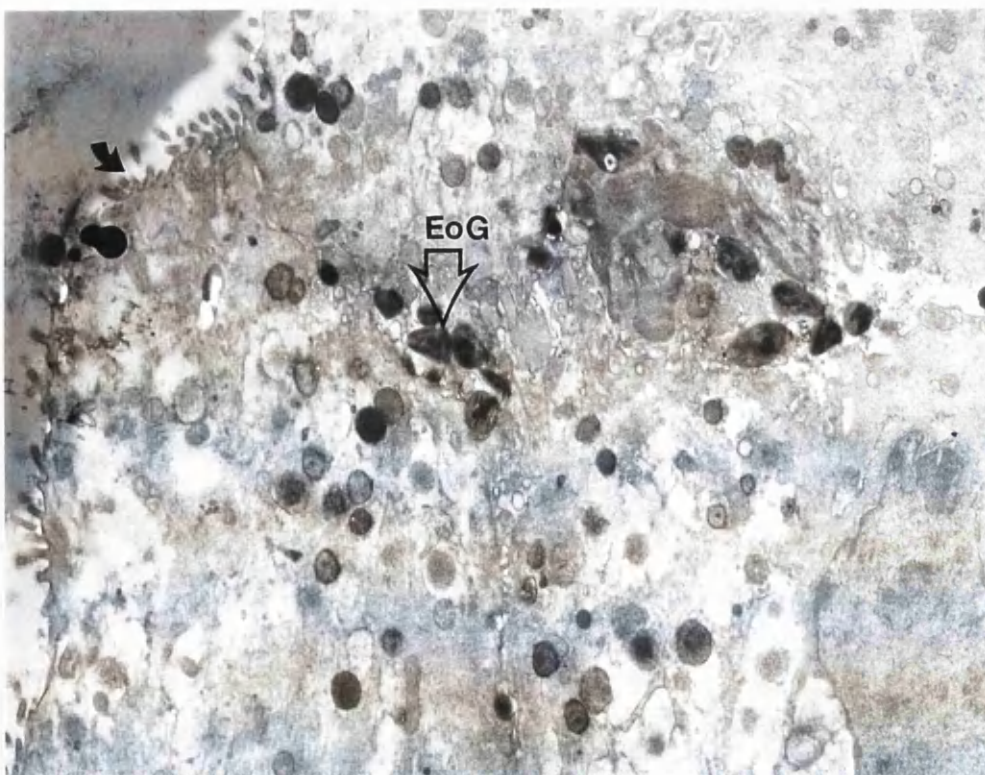
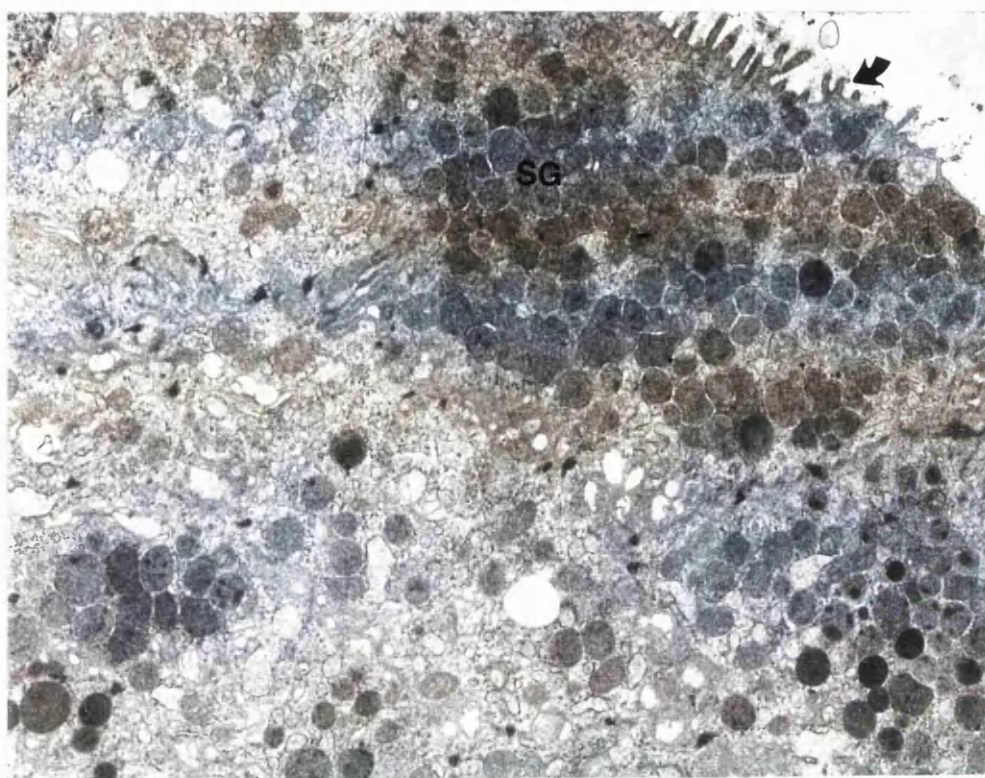


Plate 3.10

Transmission electron micrographs of the endocervix at the external os

Upper Control animal showing dense collagen (Co) and a blood vessel (BV) with a few RBCs Magnification x5.4K

Lower Treated animal showing less densely arranged collagen (Co), extravascular RBC and an enlarged blood vessel with presence of a red blood cells, platelets (Pt) and an eosinophil (Eo). Magnification x5.4K

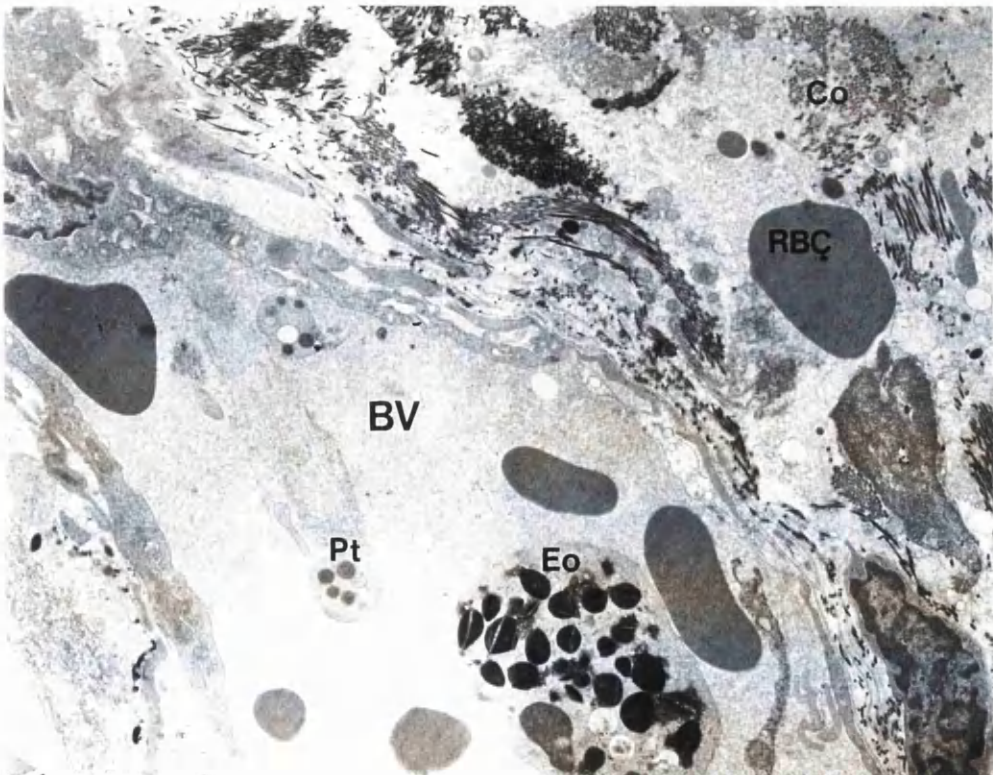


Plate 3.11

Transmission electron micrographs of the endocervix at the external os comparing control with treated.

Upper Observe the density of the collagen fibres (Co).

Magnification x8K

Lower An eosinophil (Eo) surrounded by a "halo of collagenolysis", ground substance with very little collagen(Co).

Magnification x8K

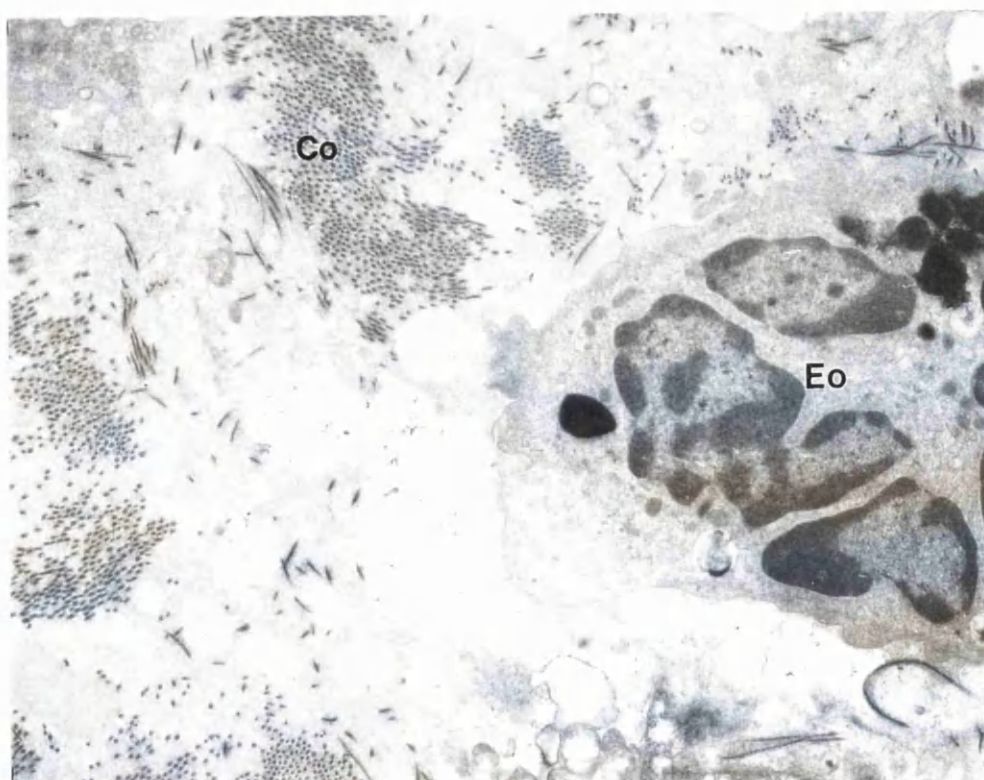
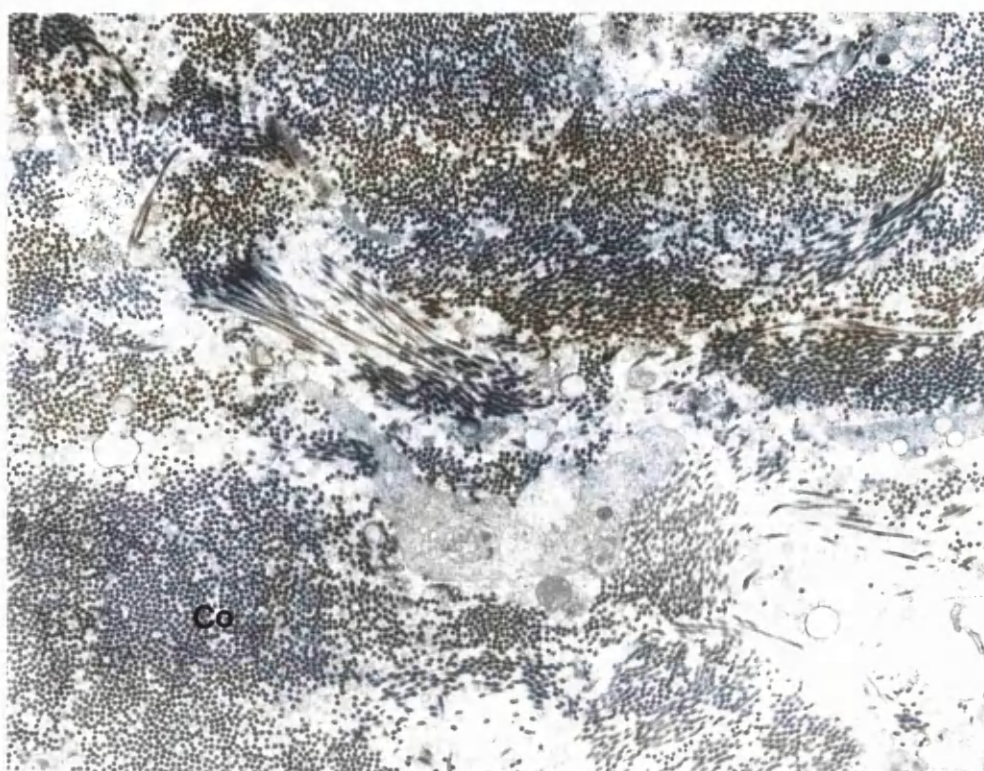


Plate 3. 12

Transmission electron micrographs of the endocervix at the external os comparing control with treated.

Upper Control animal showing a blood vessel (BV) and surrounding connective tissue with collagen (Co).

Magnification x8K

Lower Note the extravasation of a PML from the adjacent blood vessel (BV) and the dispersion of collagen fibres (Co) in this treated animal when compared with control. x8K

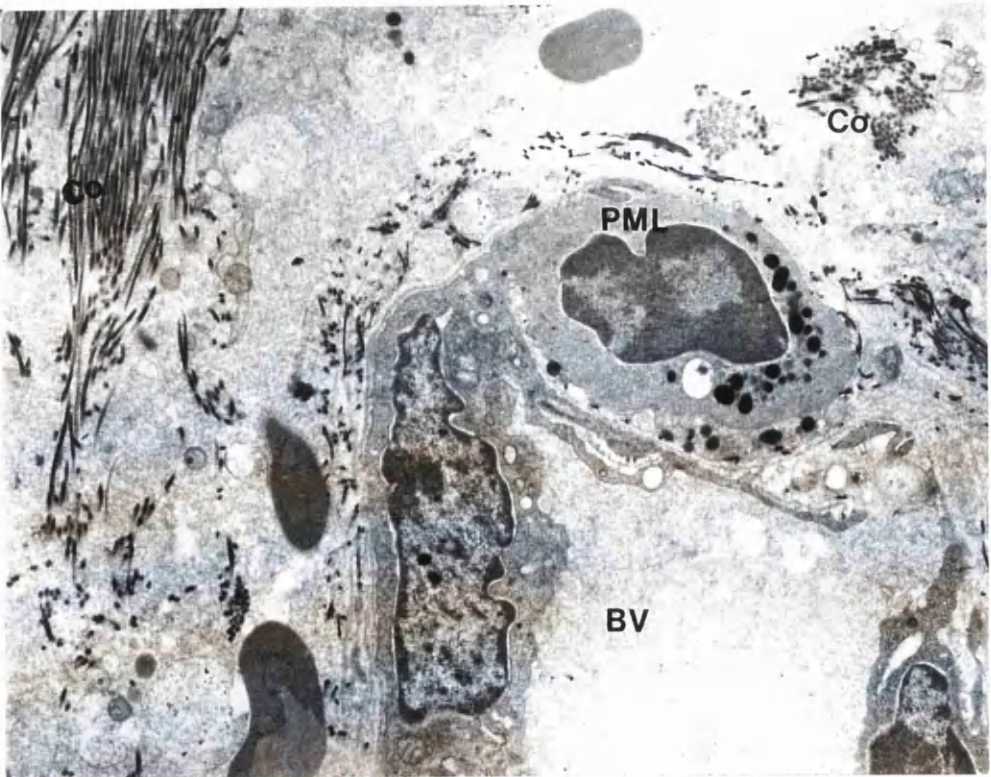
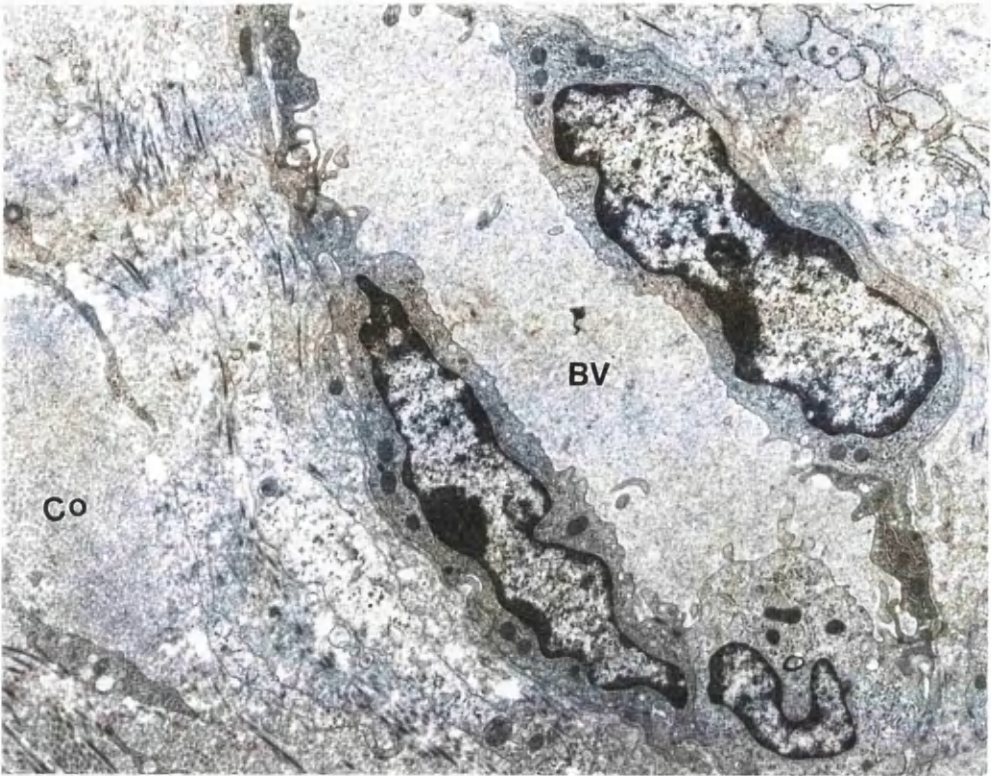


Plate 3.13

Transmission electron micrographs of the endocervix at the external os in treated sheep.

Upper Extravasation of PMLs and red blood cells (RBC) and adjacent blood vessel (BV). This finding was only present in treated animals. Magnification x9K

Lower Eosinophil aggregation, in this field, 3 cells can be observed. Note the separation of collagen fibres (Co) around the eosinophils and the presence of a fibroblast (Fb). x5.4K

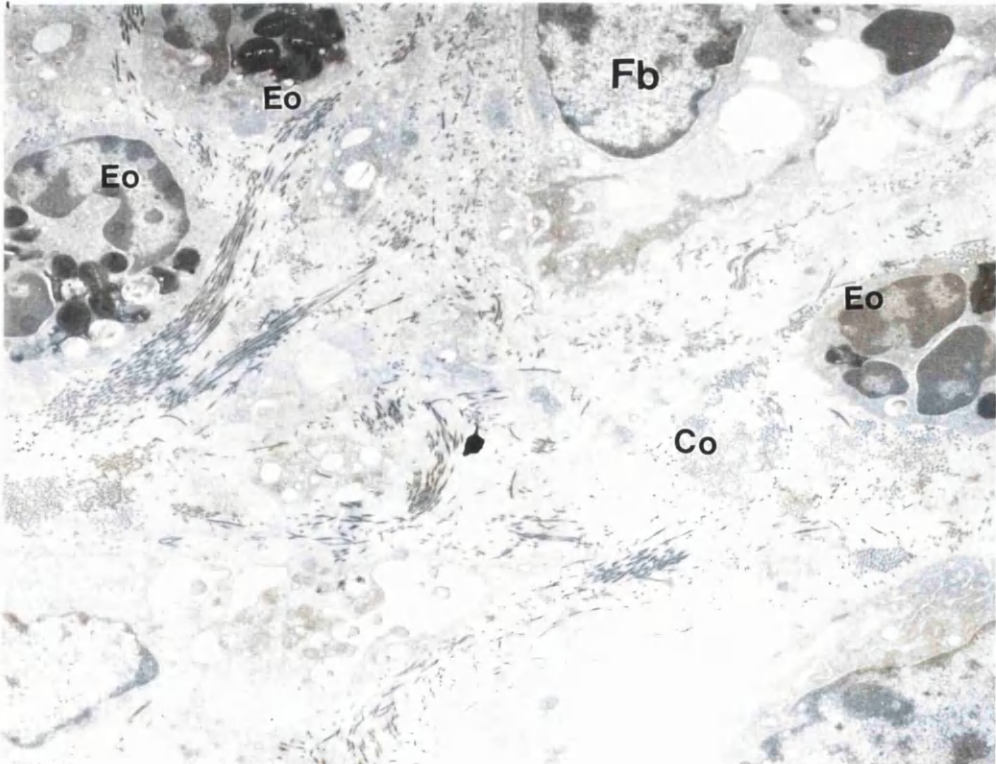
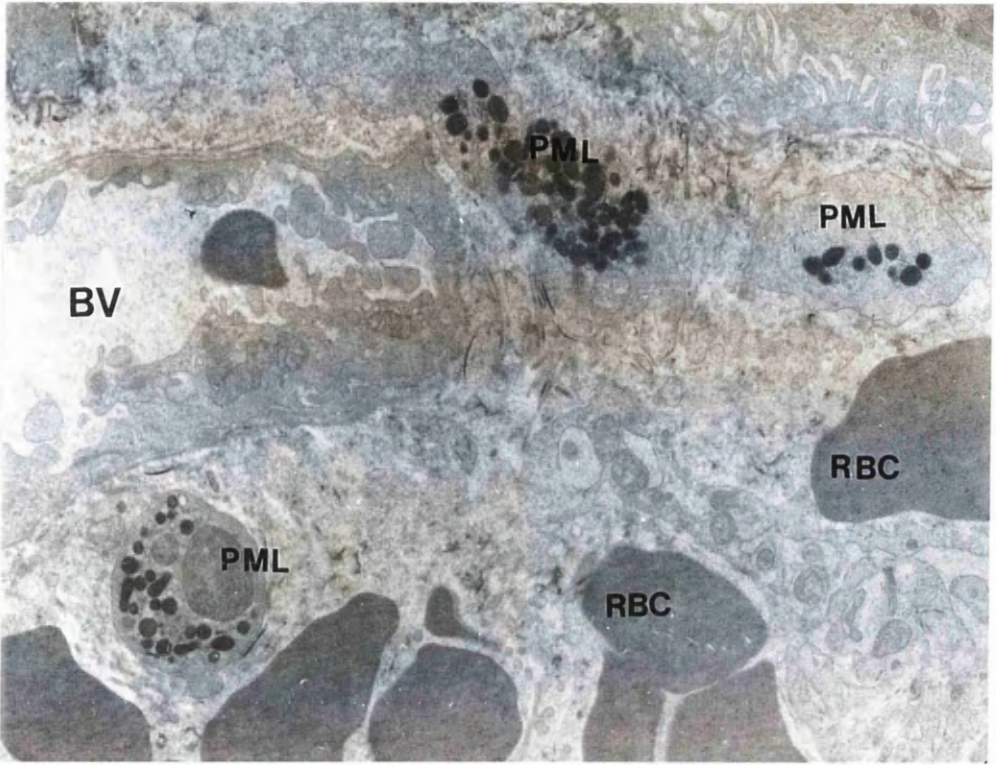


Plate 3.14

Light micrographs of the mid cervix

Upper Control animal showing a columnar epithelium (arrow).

Magnification x20

Lower Treated animal showing an inflammatory response in the epithelium (open arrow). White blood cells, red blood cells (stained in yellow) and desquamation (closed arrow). Presence of mucus (Mu) that contains red and white cells.

Magnification x20

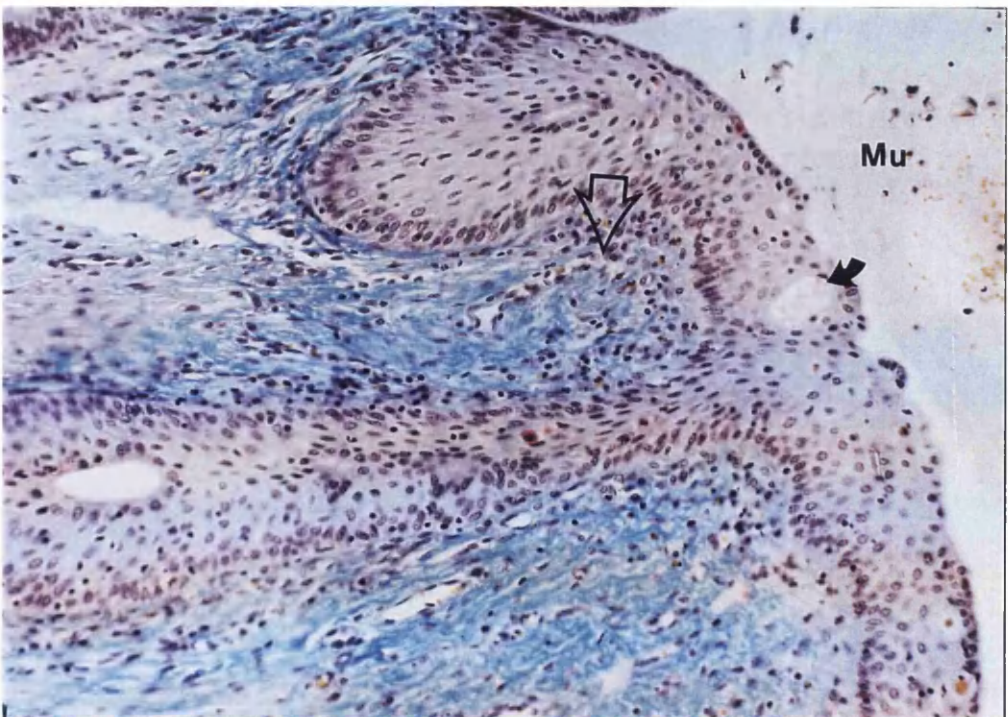
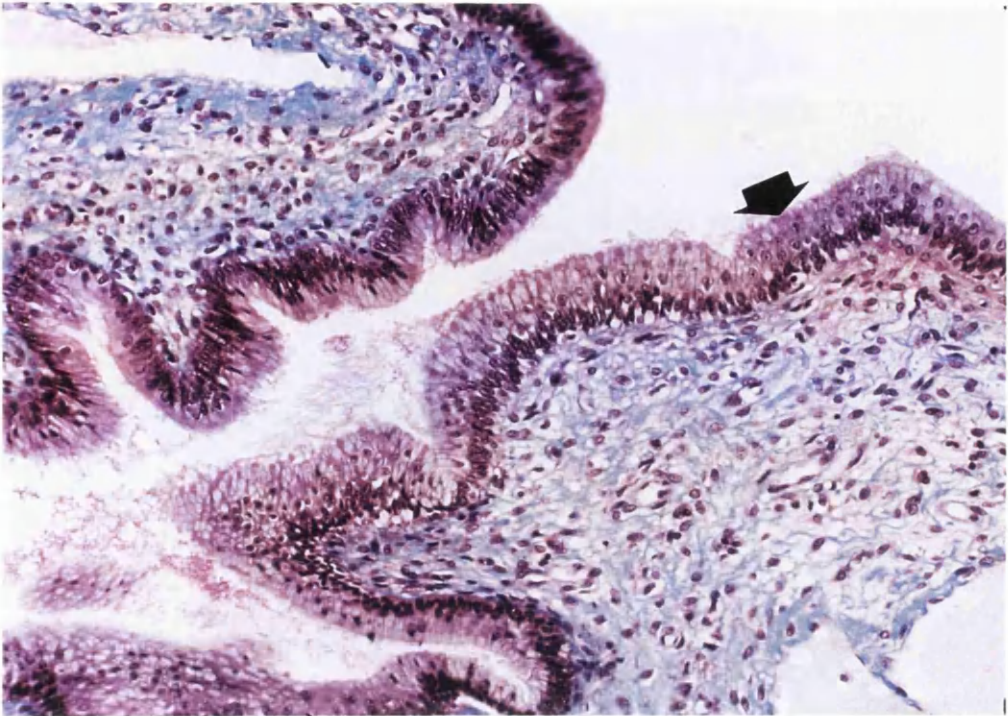


Plate 3.15

Scanning electron micrographs of the mid cervix

Upper Control animal showing a columnar epithelium with secretory (sc) and ciliated cells (cc).

Magnification x5625

Lower Treated animal showing some inflammatory cells (arrows) in the epithelial surface apart from the secretory (sc) and ciliated (cc) cells.

Magnification x5625

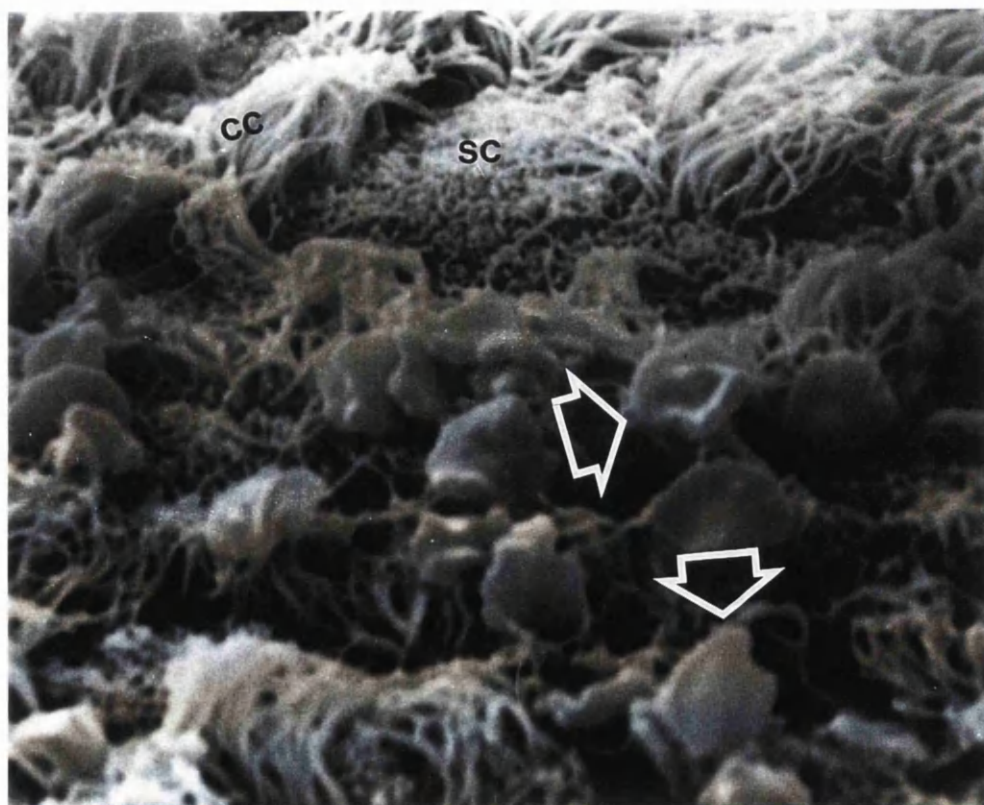
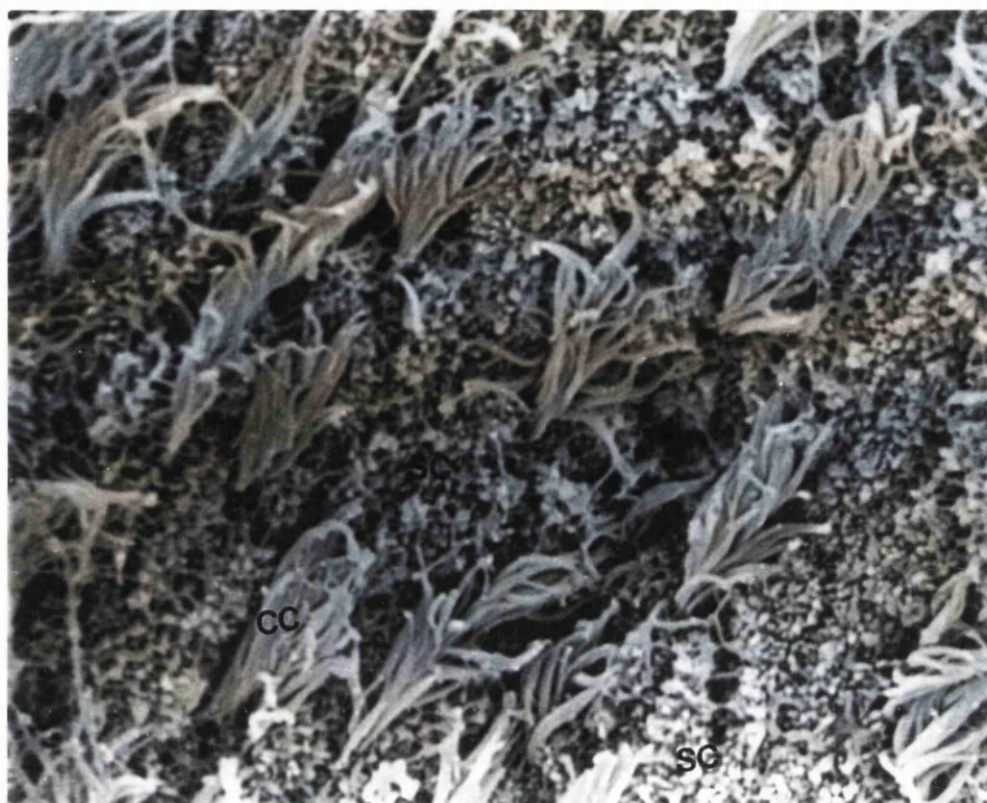


Plate 3.16

Transmission electron micrographs of the endocervix of the mid cervix

Upper Control animal showing dense collagen (Co) and smooth muscle cells (SM), these are more prominent cranially.

Magnification x13.5K

Lower Treated animal showing a similar appearance as the control group, collagen (Co) and smooth muscle cells (SM).

Magnification x13.4K

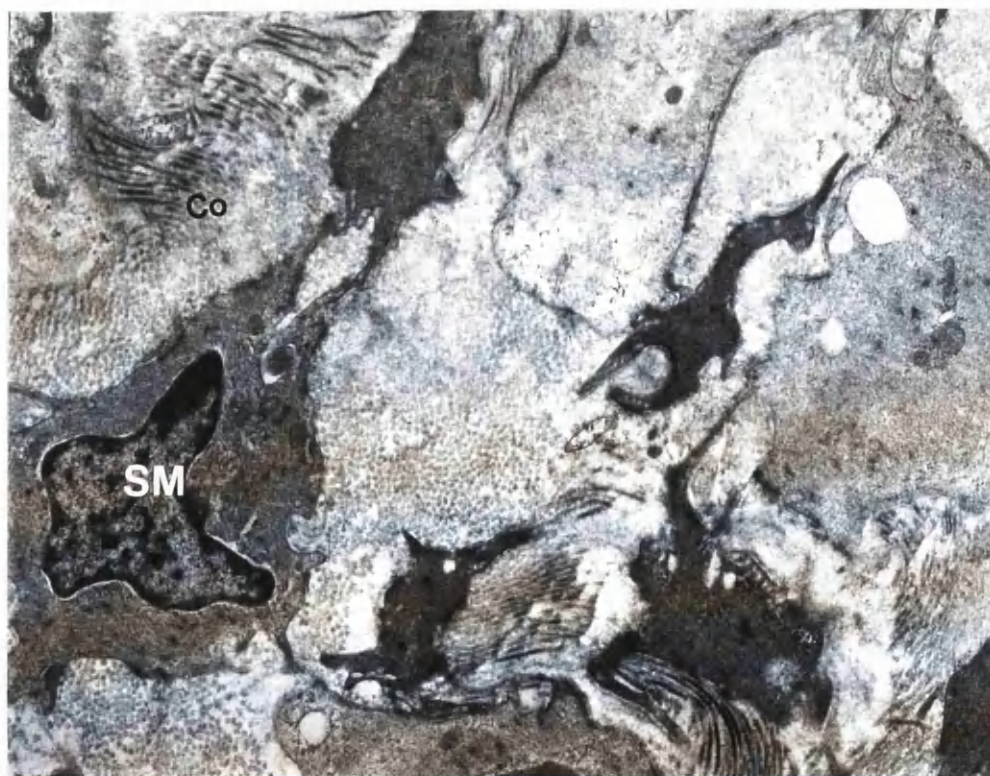
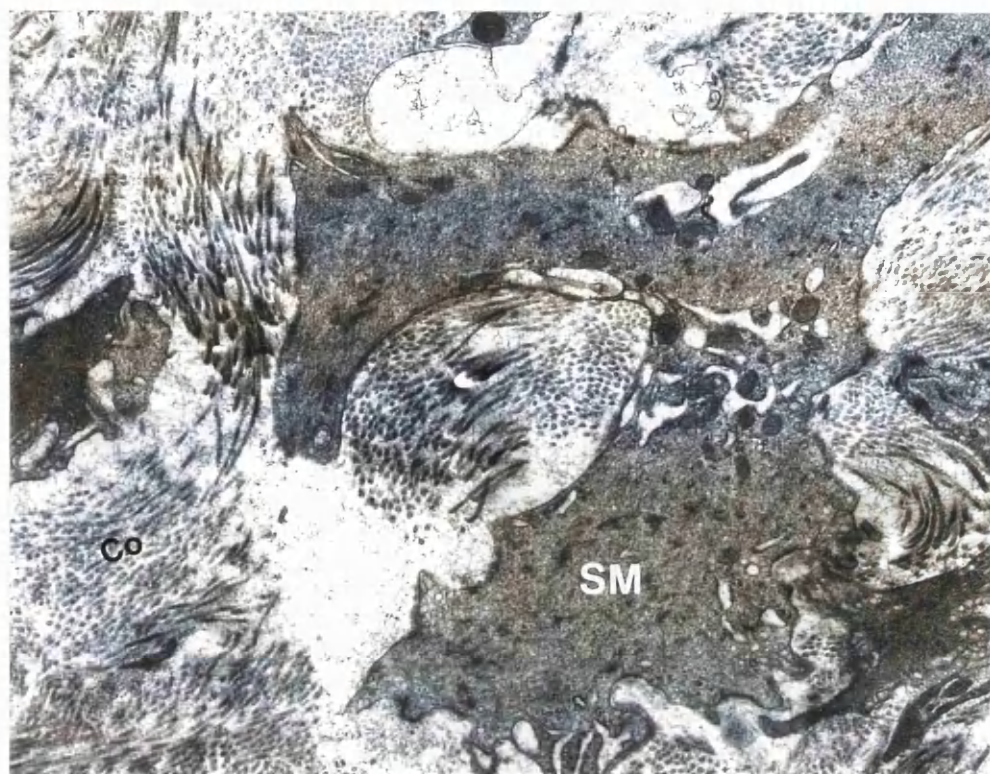


Plate 3.17

Light micrographs of the internal os of the cervix

Upper Control animal showing a columnar epithelium (arrow).

Magnification x20

Lower Treated animal showing a similar epithelium (arrow) compared to control animals. No inflammatory reaction was observed in this part of the cervix. Magnification x20

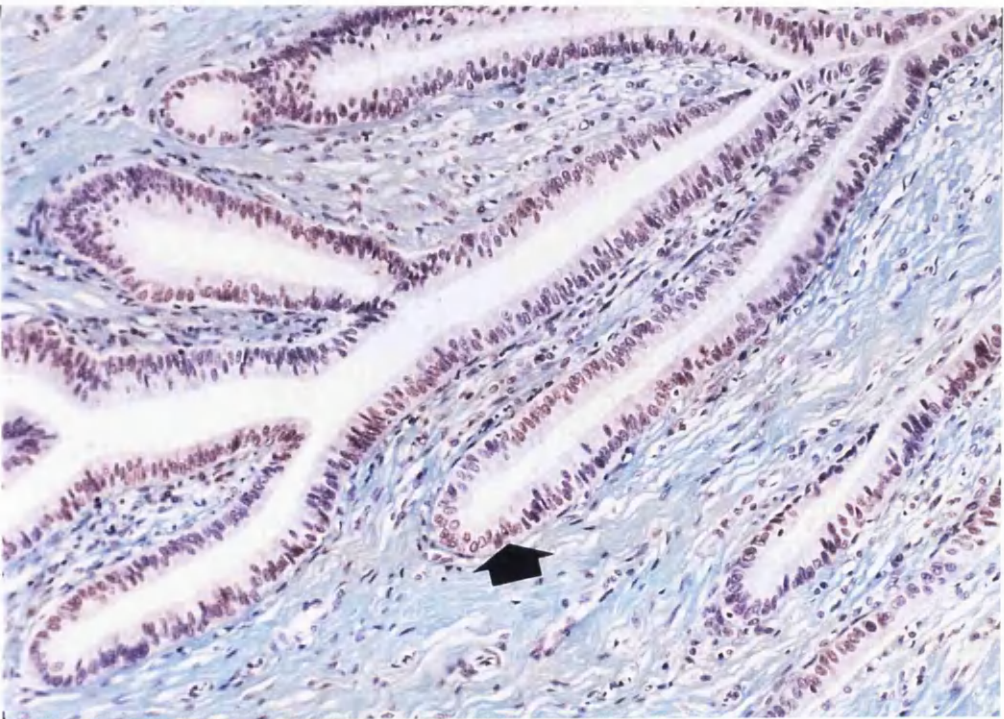
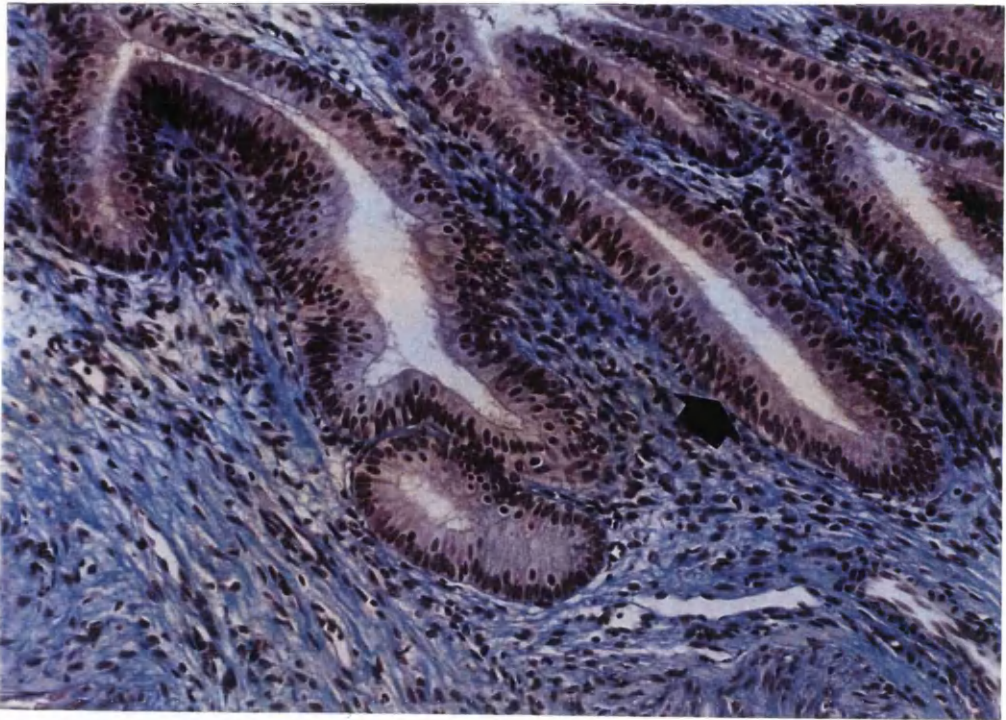


Plate 3.18

Scanning electron micrographs of the internal os of the cervix

Upper Control animal showing the typical columnar epithelium of this region with secretory (sc) and ciliated cells (cc). x6250

Lower The epithelium in treated animals was not different from controls in this portion of the cervix. Note the secretory (sc) and ciliated cells (cc) x6250

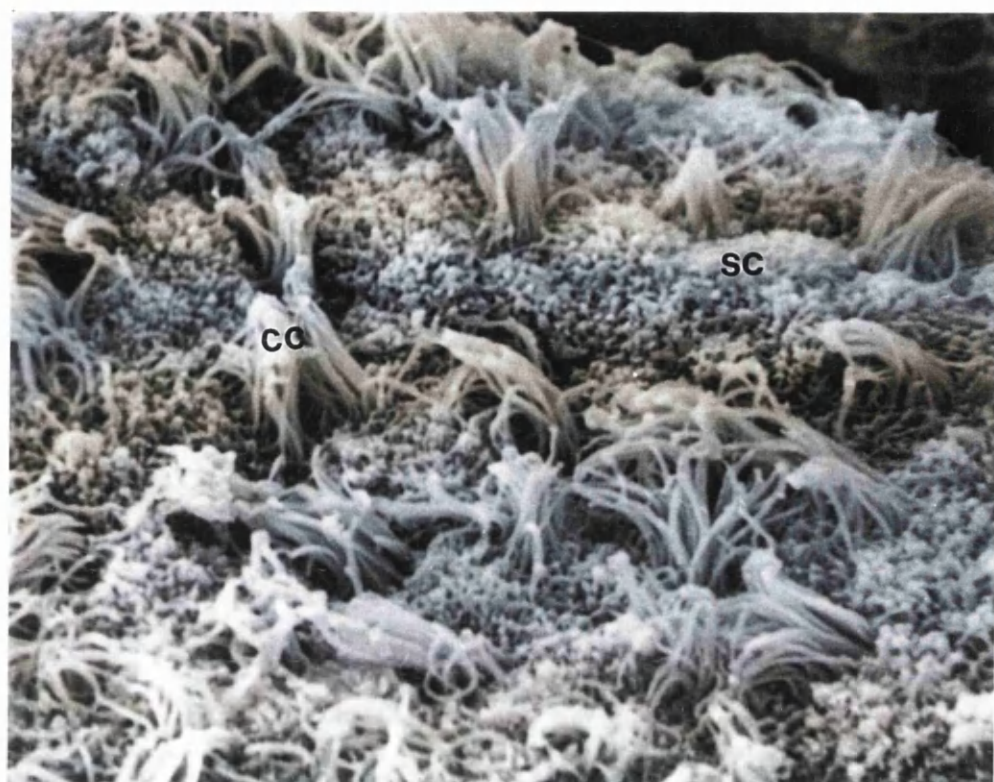


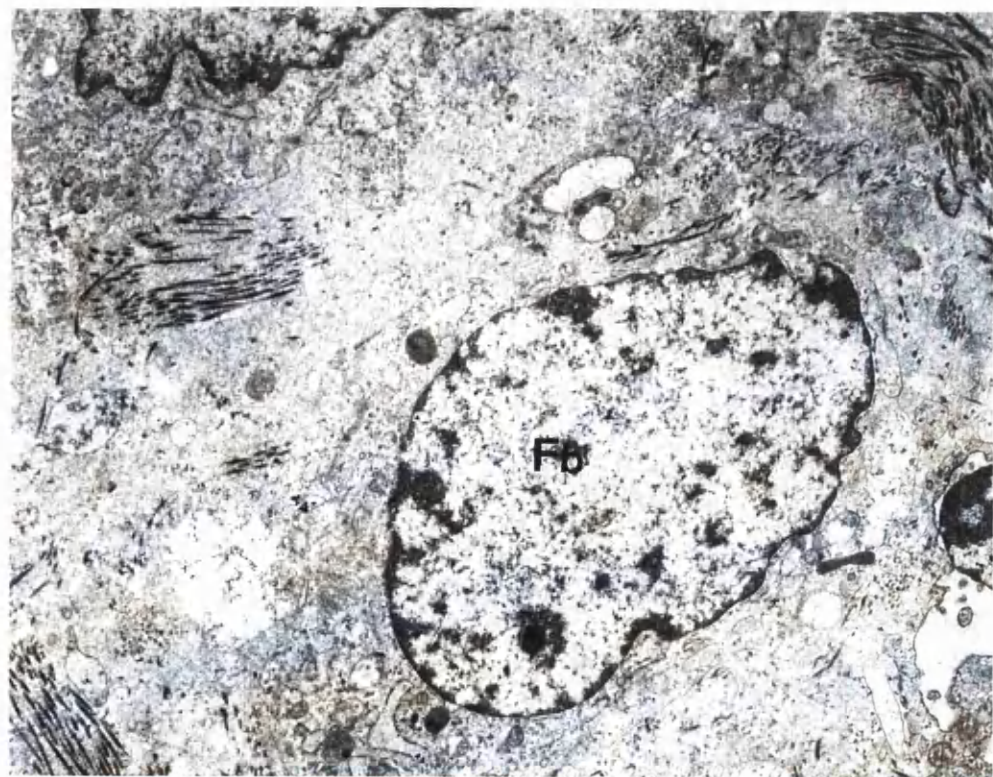
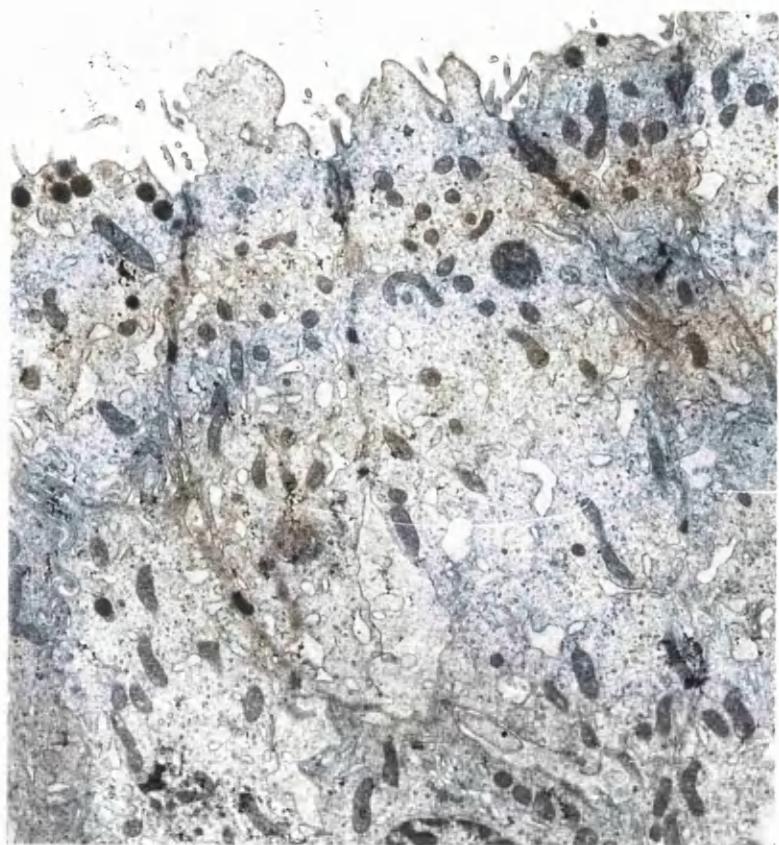
Plate 3.19

Transmission electron micrographs of the endocervix at internal os

Upper No inflammatory response was observed in the epithelium of treated groups at this site of the cervix.

Magnification x8K

Lower The subepithelium of treated animals without presence of white cells. note the presence of a fibroblast (Fb) x8K



3.4 DISCUSSION

A suitable PGE₂ regime for transcervical ET in sheep: route, form and dose.

Partial catheterization of the cervix was achieved using two vaginal preparations of PGE₂, indicating a degree of softening of the external os of the cervix. Microscopic examination of the vagina and the cervix showed collagen separation in the cranial vagina and around the external os of the cervix, but dense collagen in the mid to internal os regions of the cervix. The cervix is much longer in sheep than in humans (i.e. 5-10 cm versus less than 1 cm) and therefore it is possible that only low concentrations of PGE₂ reached the mid cervix and internal os in the present experiments. In pregnant sheep, vaginal PGE₂ is more effective on the caudal cervix, it is suggested that the dosage reaching the uterus is lower than in women when using vaginal or intracervical preparations (Fitzpatrick and Liggins, 1980; Stys et al., 1981). The pregnant sheep cervix is much longer than during the cycle, with lengths of 17.5-29 cms (Aughey, unpublished results).

Partial catheterization using an intravaginal suppository was also observed in anoestrous sheep by Rickord and White (1988), these authors proposed using the intracervical route as an alternative. However due to the difficulty of catheterising the cervix during the cycle, the administration of prostaglandins by this route is limited. Catheterization up to 4-5 cm, achieved using PGE₂ intravaginally in some cases in the present study, may be enough to reach the uterus in some breeds of sheep with short cervixes, where a mean of 5-6.7 cm has been reported during the cycle (Ward, 1968; Halbert et al., 1990a). Most anatomy text books also talk about lengths of 4-6 cm (Sisson, 1975; Habel, 1989). The incomplete catheterization of this study may be explained by the long cervix observed in non-pregnant Scottish

Greyface (length of 10 cm). It may be worth looking at breed differences in the length of the cervix *in vivo*, for example using ultrasonography (see chapter IV).

For these reasons, the use of PG analogues administered by general routes may be more appropriate in sheep breeds with "long cervices". In addition, these compounds are more potent than the natural PGE₂ with lower side effects generally, therefore being preferred as abortifacients in early pregnancy and during the cycle in women (Bygdeman, 1992). Sulprostone could be injected intramuscularly or intracervically, apparently the later route is well tolerated by women (Wiechell, 1979; Lueken and Lindermann, 1979). Misoprostol by the oral route is another alternative, additional advantages are the lower price of this drug and that it does not require refrigeration (see chapter I). Drugs administered by the oral route in ruminants however require a full investigation of possible interferences with the ruminal contents, to achieve an effective treatment and not to upset the normal digestion (Jenkins, 1988).

An additional problem of using intravaginal pessaries here was that some ewes (5/14) lost them. The intravaginal gel (used in trial 2) was a viscous preparation and therefore this was not a problem. The gel comes in a syringe, the nozzle had to be extended 6 mm with endotracheal tube to reach the external os in the sheep. The author suggests that an extended embolus may be also helpful in order to push all the gel out of the syringe.

Doses used in this work were those recommended by medical pharmaceuticals and are the same as those used during pregnancy in sheep (Fitzpatrick and Liggins, 1980; Stys et al., 1981). It is possible that the second pessary could be more effective when administered 12 hours after the first, because the whole dose was probably released by then at a rate of 1 mg per hour. Here a 24 hours interval was chosen instead, for

convenience, in order to accommodate the evaluation of the PGE₂ effect with staff working patterns.

2. Mechanism of action of PGE₂ during the cycle

These morphological studies have shown for the first time that PGE₂ induces an inflammatory response on day 6 of the cycle and that in addition to pregnancy, it can modify the connective tissue of the cervix at this stage. In the next section the possible role of the inflammatory cells observed in treated groups here will be discussed.

The PML and fibroblast

The observation of PML activation following PGE₂ treatment here, is consistent with the idea that PML degranulation is involved in PGE₂-induced cervical ripening supported by the evidence of a rapid and transitory increase in the activities of collagenase and elastase in women (Osmers et al., 1991b; Rath et al., 1993).

It has been suggested that PGE₂ could exert its proinflammatory effect in the cervix and in the skin by acting as a vasodilator on EP₂ and EP₄ receptors of the smooth muscle of blood vessels. This action is synergistic with IL-8 which activates the PMLs. IL-8 is suppressed by progesterone during pregnancy and increases at labour with the progesterone withdrawal (Kelly, 1996). The action of PGE₂ during the cycle (under the influence of high progesterone and therefore in the absence of other proinflammatory mediators) may be better explained by a direct action of PGE₂ on PML receptors (Armstrong, 1992).

Fibroblasts, rather than fibrocytes were also observed and therefore it cannot be ruled that the fibroblast could be another source of collagenase (Goshowaki et al., 1988). However the role of this cell is unclear because, although PGE₂ and cAMP induce the synthesis of mRNA for proMMP-1 in

fibroblasts of other tissues, in the cervical fibroblasts the administration of cAMP inhibited mRNA synthesis of proMMP-1 and proMMP-3 (Takahashi et al., 1991).

Presence of activated eosinophils in PGE₂-treated ovine cervix

Apart from the PMLs, here it was observed an increased number and degranulation of eosinophils following PGE₂ treatment. This is consistent with observations made during physiological and PGE₂-induced ripening in late pregnant sheep (Liggins et al., 1981; Fosang et al., 1984; Fitzpatrick and Liggins, 1980).

Eosinophilic granules may be sources of collagenolytic enzymes or cationic proteins. Haloes of collagenolysis have been observed here and by others in late pregnancy (rat: Luque et al., 1989; women: Knudsen, 1991). Like the PML, eosinophils may store several proteinases, including leukocyte elastase (Lungarella et al., 1992), a collagenase that degrades the main collagen types of the cervix, namely type I and III (Hibbs et al., 1982) and gelatinase B (Stähle-Bäckdahl and Parks, 1993). This however remains as an hypothesis and it is unknown whether collagenase and elastase are released from eosinophilic granules during cervical ripening. Furthermore, recent reports are questioning the presence of elastase in the eosinophil granules (Wiedow et al., 1996). Release of cationic proteins during degranulation is another possibility, eosinophilic major basic protein has been observed during cervical ripening in rats (Duchesne and Badia, 1992).

Platelets and mast cells

Eosinophil activation involves an increase in the intracellular calcium levels and so far only negative responses to PGE₂ (involving increases in cAMP) have been described (Giembycz and Barnes, 1993). The eosinophil activation by PGE₂ treatment observed here may be an indirect action on other cells that can be activated by the EP₃R, such as the PML, mast cells or platelets (Wheeldon and Vardey, 1993; Nishigaki et al., 1993; Mathews and Jones, 1993; Kunapullil, 1994; Blockmans et al., 1995). Mast cells have been suspected to be involved in the activation of eosinophils during physiological cervical ripening (Knudsen, 1991). Our results are also consistent with this view while also suggesting a possible role for platelets in PGE₂-induced ripening.

Platelet aggregation was observed in blood vessels near eosinophils and PMLs. Other signs of platelet activation, changes in shape and degranulation, were not seen. Platelets are sources of cytokines for eosinophils as discussed earlier (Herd and Page, 1995). Degranulating mast cells were observed, this is consistent with observations during physiological (sheep: Aughey, personal communication; human: Lo Stumbo, 1970) or IL-induced, and antiprogestagen-induced cervical ripening (Hegele-Hartung et al., 1989; Chwalisz et al., 1994). The role of this cell in cervical ripening has been suggested to be the recruitment and activation of eosinophils (Knudsen et al., 1991). They can liberate chemotactic factors for eosinophils and PMLs and they are considered the initiators of acute inflammatory responses (Galli, 1993; Galli and Costa, 1995; Galli and Weshil, 1996). They also contain very large amounts of tryptase (in humans) and chymase (in murines), which participate in extracellular matrix degradation in other tissues (Caughey, 1994).

Macrophages

The macrophages seen in the present study appear to contain eosinophilic particles in them (Plate 3.6). This has also been described in other situations, like oestrus, asthma and other inflammatory responses (Ross and Klebanoff, 1966). Macrophages/monocytes are known to be involved in the phagocytosis of apoptotic eosinophils and PMLs, thus removing the detritus of the inflammatory reactions (Savill and Haslett, 1995). This role is consistent with the observations of this study.

It has recently been reviewed that PGE₂ is crucial for the production of metalloproteinases by macrophages (Goetzl et al., 1996). Besides, monocytes also contain an elastase that is cross-reactive with the PML enzyme and it is lost in mature macrophages. Unlike the eosinophilic enzyme, the presence of HLE in monocytes, appears to be well established (Kargi et al., 1990; Campbell et al., 1991c; Caughey, 1994). It is not surprising that a hypothetical role in the degradation of the cervical extracellular matrix by these cells in PGE₂-induced cervical ripening has been suggested (Rayburn et al., 1994). The morphological studies carried out here neither support nor discard this hypothesis.

Other authors have reported increased numbers of macrophages in physiological and other drug-induced cervical ripening (Fosang et al., 1984; Hegele-Hartung et al., 1989; Chawalisz et al., 1994). Macrophages are the most important source of PGE₂ in inflammatory reactions (Coleman et al., 1990; Corcoran et al., 1995). Another possible role of this cell in physiological ripening may be the synthesis of PGE₂ which could explain why here, where the PGE₂ was administered exogenously, increased numbers of these cells were not observed.

Red blood cells (RBCs) and blood vessels

As in this study, extravasation of RBCs has been observed during physiological ripening by Fosang (1984). This could be due to elastase activity, inducing vascular damage and causing haemorrhage, this has been observed in the lung (Fletcher et al., 1995). These authors prevented the haemorrhage by inhibiting the activity of leukocyte elastase. The role of RBCs in cervical ripening, if any, has not been discussed. Red blood cells within blood vessels participate in the margination of leukocytes, which is the start of extravascular migration (reviewed by De Jongh et al., 1996). Recent work however, showed that PGE₂ can have an effect on RBCs enzymes, suggesting that PGE₂ could promote oxygenation of the tissue (Neves et al., 1996). Certainly, binding sites for PGE₂ have been reported in the RBCs of many tissues (*corpus luteum*, uterus and peripheral blood) and vertebrate species (bovine, frog and human: Robertson, 1986; Chegini et al., 1986; Rao, 1990).

Vasodilatation was observed here following PGE₂ treatment. For many years this was thought to be the only proinflammatory effect of PGE₂ (Liggins, 1981). PGE₂ could cause vasodilatation by several mechanisms: 1) relaxing the smooth muscle of blood vessels, 2) acting on endothelial cells or 3) acting on PMLs, which in turn cause exudation (Armstrong et al., 1995).

Epithelial changes as part of the inflammatory response

An increase in the production of mucus, desquamation and keratinization of cells in the epithelium occurred following PGE₂ treatments. These changes may be also part of the inflammatory response. In other non-reproductive mucosae, the epithelium also participates in the inflammatory response. One example of this is the denudation of the bronchial epithelium in asthma and may be due to cationic proteins of the eosinophils and/or nitric oxide (Butterfield and Leiferman, 1993; Galli and Costa, 1995). There are different

inflammatory mediators, produced by the white cells mentioned earlier that can increase the secretion of mucus: cationic proteins from the eosinophil, the serine proteinases chymase, leukocyte elastase and cathepsin G (Venge, 1993; Caughey, 1994).

Summary

The findings were consistent with the induction by PGE₂ of an acute inflammatory response, characterised by 1) edema, 2) leukocyte migration of the acute type (mainly PMLs and eosinophils without lymphocytes and macrophages) and 3) short duration and the reversibility of the inflammatory response after a week. Resident cells of the connective tissue, such as mast cells and fibroblasts also participated. Evidence of cell activation was observed: 1) PML degranulation and aggregation, 2) eosinophil degranulation, 3) platelet aggregation and 4) mast cell degranulation. Inflammatory reactions in the mucosal surfaces characterised by hypersecretion of mucus and desquamation or denudation of the epithelium were also observed here.

During physiological ripening collagenase, gelatinase and leukocyte elastase activities appear to be crucial for the softening of the cervical tissue by breaking down collagen fibres and other components of the extracellular matrix. There is now good evidence that these activities can be attributed to the degranulation of the PML, with the release of MMP-8, MMP-9 and leukocyte elastase (Kanayama and Terao, 1991; Osmers et al., 1995). The PML is the only known source of MMP-8 (Woessner, 1994) and the main source of leukocyte elastase (Doherty and Hanusz, 1994). PMLs must be activated to release these enzymes and there is evidence of activation during labour (Okamura et al., 1988; Kanayama and Terao, 1991; Osmers et al., 1992; Owiny et al., 1995) and also that PMLs activators like interleukin-8 (IL-8) and the bacterial substrate (N-formyl-methionyl-leucyl-phenylalanine

FMLP) (Baggiolini et al., 1992; Baggiolini et al., 1993) can induce ripening (El Maradny et al., 1994 and 1995).

In PGE₂-induced cervical ripening, the same enzymatic activities as in physiological ripening have been found, i.e. collagenase, gelatinase and elastase (Osmers et al., 1991b; Rath et al., 1993; El Maradny et al., 1996). The cell origin is not yet clear but the rapid release of collagenase and elastase strongly supports the role of enzymes stored in PMLs (Osmers et al., 1991b; Rath et al., 1993). Elastase has been localised in PMLs and not in other leukocyte following treatment with Gemeprost (Geer et al., 1992). The release of collagenase is transitory, activity cannot be found 24 hours after treatment (Osmers et al., 1991b; El Maradny et al., 1996), this may also exclude a role of an induced MMP-1 by fibroblasts and macrophages. In contrast, the release of MMP-1 is maximal following a 24 hour incubation in those cells (Corcoran et al., 1992; Rajabi et al., 1990). In conclusion it is an attractive hypothesis that PGE₂ could induce cervical ripening by activating PMLs. Activation responses should include chemotaxis, degranulation and release of oxygen intermediates, to explain the presence of these cells in the cervix, the release of MMP-8, MMP-9 and leukocyte elastase and the activation of these proteinases respectively (Doherty and Hanusz, 1994). Chemotaxis and degranulation were observed here, other responses and the release of enzymes need further investigation.

CHAPTER 4

STUDIES OF THE OVINE PROSTAGLANDIN E₂ (PGE₂) RECEPTOR (EP₃R) cDNA.

4. 1 INTRODUCTION AND AIMS

The purpose of this thesis is to gain some understanding of the sensitivity of the ovine cervix to the ripening effect of PGE₂ during the luteal phase, which could allow the development of pharmacological regimes for transcervical AI and ET. This chapter deals with a possible approach to this problem, which is the study of the presence of PGE₂ receptors in the ovine cervix at different reproductive stages. The sensitivity of the cervix during the cycle appears to be lower than during the prepartum stage in sheep and women (Owiny and Fitzpatrick, 1990; Mylne et al., 1992; Uldbjerg and Ulmsten, 1990; see also chapter III). These differences may be due to the endocrine and paracrine environment in which PG have to act. During the luteal phase and due to the negative influence that progesterone has in cervical ripening, the lowest sensitivity may be expected.

The effects of PGE₂ in different tissues, including the reproductive tract and the immune response, are very complex and often contradictory. Recent studies have shown the existence of at least four receptor subtypes (EP₁-EP₄) and several isoforms for the EP₃ subtype. It is anticipated that the diversity of receptors found, will explain the actions of PGE₂ in different tissues and cells and will make possible the development of more specific drugs to control these actions (Narumiya et al., 1993). This may be also true for the understanding of the PGE₂ role in cervical ripening and the development of new PGE₂ analogues for this purpose.

Prostanoids are formed locally and act on specific G protein linked receptors in a paracrine manner in several tissues (Coleman et al., 1994). Similarly, previous studies support the existence of PGE₂ receptors in the cervix, since they have demonstrated local synthesis of PGE₂ by the cervix, a pharmacological action of local PGE₂ preparations, the existence of PGE₂

binding sites and second messenger responses *in vitro* to the administration of PGE₂ (Ellwood et al., 1981; Noström et al., 1983; Adelantado et al., 1988; Rao, 1990; Uldbjerg and Ulmsten, 1990). But it is unknown which receptor subtype(s) may be present in the cervix or which cell(s) bear the receptors. At the beginning of these studies, antibodies were unavailable for any of the four prostaglandin E₂ receptor subtypes (EP₁-EP₄) and the only sequence available was from cDNA cloned for two subtypes in a few mammalian species, the EP₃ receptor and the EP₂ receptor that later resulted to be in fact the EP₄ receptor (see chapter I). In the absence of ovine reagents for the detection or quantification of these receptors, the study of the EP₃R cDNA was chosen as a first step in the study of the PGE₂ receptors (EPs) in the cervix. Pharmacological approaches are less sensitive or specific, since complete agonist and antagonists do not exist for the four EP receptors. Another limitation of that approach is that the isoforms of the EP₃R cannot be differentiated either (Coleman et al., 1990; Coleman et al., 1994).

The 4 receptor subtypes (EP₁-EP₄) and the EP₃R isoforms are functionally different, and the investigation of their involvement in cervical ripening is probably interesting in all the cases, but it is a too big task for a PhD chapter. In this thesis the EP₃R was chosen because its cDNA was better known than the other three subtypes and its function appeared to be compatible with current theories about the physiological cervical ripening. Some commercial PGE₂ analogues frequently used for cervical ripening (Bygdeman, 1992) are potent EP₃ agonists in other tissues (Coleman et al., 1990; Qian et al., 1994). It is also interesting that the EP₃R may be implicated in the activation of PMLs. Namely on chemotaxis and release of LTB₄, which is in itself a potent activator of other PMLs and immune cells (Armstrong, 1992; Wheeldon and Vardey, 1993). An attractive hypothesis

therefore, is that the EP₃R may mediate the cervical ripening effect of PGE₂ and its analogues by priming the PML. Local administration of PGE₂ during pregnancy induces a rapid increase in the activity of collagenase and elastase, which is probably due to the degranulation of PMLs (Rath et al., 1993). In physiological ripening there was also evidence of PML activation, with release of collagenase, elastase and oxygen mediators (Junqueira, et al., 1980; Parry and Ellwood, 1981; Kanayama and Terao, 1991; Osmer et al., 1992). The degranulation of PMLs following PGE₂ administration (observed in the previous chapter), is also compatible with this hypothesis.

In contrast, the literature in other disciplines is dominated by literature on the negative effects of PGE₂ on all the aspects of PML priming. This action may be mediated by an increase in cAMP, since the direct activation of cAMP by other agents has an inhibitory effect on PML priming (Reibman et al., 1990; Harvath et al., 1991; Gryglewsky and Salvemini, 1992). The PGE₂ receptors coupled positively with cAMP are the EP₂ and EP₄, which may be inhibitor receptors for PML functions, and from this point of view, these receptors may not be of interest in the study of cervical ripening. Recent evidence has shown that PGE₂ is not only coupled positively with cAMP, but also with increases of calcium in the cell, through the EP₃ receptor (Coleman et al., 1994) and the increase of intracellular calcium by other agents can actually prime the PML (Baggiolini et al., 1993). At the beginning of these studies, the second messenger of the EP₁ subtype, the sequence and its possible participation in PML functions were completely unknown, which also contributed to the choice in this thesis to study the EP₃R.

Alternative splicing occurs at the 3' end of the EP₃R cDNA of several mammals studied. Therefore, the initial aim of this work was to isolate and sequence EP₃R 3'end cDNAs, using an RT-PCR (RACE) method and

bovine primers, designed from conserved areas. The objectives being to obtain sequence information and to perform mRNA expression studies of the isoforms of the EP₃ receptor. A semiquantitative assay would then allow study of mRNA expression of the different receptor isoforms in cervical tissue collected at different stages (oestrus, early luteal and peripartum).

As the work progressed, the objectives were redefined. A method technically more simple, using two bovine primers in a normal RT-PCR, would be used to:

- 1) Isolate and sequence a fragment of cDNA encoding for the ovine EP₃R
- 2) Study the mRNA expression pattern by northern analysis or the more sensitive method, RT-PCR and to identify a rich source for the cloning of this receptor using RACE
- 3) Study the presence and the quantity of mRNA expression in cervical tissue collected at different reproductive stages (oestrus, early luteal and peripartum), using quantitative northern analysis, RT-PCR or Southern analysis, depending on the sensitivity required.

4.2 EXPERIMENTAL PROTOCOLS

4.2.1 RACE using mRNA from mouse testis

Initially cDNAs expressed in the mouse testis were used. This involved steroidogenic enzymes like cholesterol side chain cleavage (CSCC) and cytochrome P450 17 α -hydroxylase, the androgen binding protein (ABP) and β -actin. The primers had been designed and used for semiquantitative studies in this laboratory by others (O'Shaughnessy and Murphy, 1993), and therefore they could be used as a well known model to test the limitations of the 3'end RACE technique, which, like in other RT-PCR experiments, are mainly the size of the expected product and the

abundance of the target sequence. The mouse testis model offered several advantages over the study of the ovine EP₃R cDNA, because the following parameters were known from previous studies: that the primers worked, that the PCR products were actually expressed in those organs, the expected sizes of the products and also the relative abundance of these products in those organs (O'Shaughnessy and Murphy, 1993)

The amplification of those cDNAs in the mouse gonads were also used to set up the optimal conditions and to control for the well functioning of the technique at later stages. The efficiency of the RT of the RACE method was compared with other RT methods (random hexamers and oligodT), by amplifying with PCR, carried out with two gene-specific primers. The limitations of the PCR of the RACE method was then studied by amplifying cDNAs of different abundances in the testis and fragments of different lengths.

4.2.2 RACE using mRNA from ovine tissues

The kidney and the uterus were selected as potential rich sources of the EP₃R message, based on mRNA studies in other mammalian species. The studies of the ovine EP₃R were initiated in the kidney because it was reported as the richest source in the mouse (Sugimoto et al., 1992). Unlike in those studies, the whole kidney could not be used for RNA extraction in sheep. Studies were initiated, using the renal cortex and later continued in the external medulla. This was based on new information showing sublocalisation of the EP₃R mRNA and EP receptors mainly in the external medulla (Takeuchi et al., 1993; Breyer et al., 1993; Sugimoto et al., 1994).

RNA was extracted from those tissues and the conditions of RACE, established in the mouse experiments, were used as a starting point to amplify with a bovine gene-specific primer. Further work was done to optimise the conditions in the ovine samples. Several parts of the kidney, two gene-specific primers and two sets of RACE primers (hybrid and adapter) were tested. Following the demonstration of EP₃R mRNA expression in several ovine tissues by RT-PCR, RACE was also performed in those tissues, using the 5' end primer as the gene specific primer.

4.2.3 RT-PCR

PCR, using bovine primers from areas conserved in other species, was used to isolate a cDNA fragment of the ovine EP₃R. This cDNA fragment was sequenced by cycle sequencing to confirm its identity as the ovine homologue of the EP₃R cDNA. The cDNA once isolated, allowed the production of probes for mRNA expression studies. Because low levels of expression were suspected, RT-PCR and Southern analysis were used to study the mRNA distribution in ovine tissues. The fragment to be amplified was intronless and therefore RNA incubations with DNase I were required before the RT-PCR to show mRNA expression.

Attempts were made to develop a semiquantitative RT-PCR assay, mRNA levels could be compared between the cervix and other tissues in which the EP₃R has a known function. Comparisons could also be made between cervixes in several hormonal contexts: oestrus, early luteal phase, and periparturient stage. Work was carried out towards the development of this assay, finding a suitable housekeeping gene and optimising the reactions.

4.3 RESULTS

4.3.1 Attempts to isolate and sequence the 3' end of ovine EP₃ receptor mRNA isoforms by RACE

Mouse studies

In the mouse testis model, the reverse transcription primer of RACE worked well, as indicated by the amplification in a normal PCR of fragments internal to the cDNA of the genes: β -actin, cholesterol side-chain cleavage (CSCC), cytochrome P-450 17 α -hydroxylase and androgen binding protein (ABP). However results were better using a conventional method for the reverse transcription, such as random hexamers, rather than the RACE primer. Different conditions for the RT (mainly concentrations of primer, DTT and dNTP) were tested in this system and the optimum conditions were those described in Materials and Methods.

Using the RACE primer with a gene specific primer in the PCR, clear bands of the expected size were only amplified in the case of cytochrome P-450 17 α -hydroxylase (1200 bp). Shorter products (1400 bp) than expected (1600 bp) were obtained with the β -actin primer, and a faint band of the expected size (650 bp) was obtained with ABP primers.

The conclusion was that the RACE method could work, but there will be limitations when attempting to amplify target sequences of low copy numbers, like ABP in the testis, and/or target sequences longer than 1200 bp. The next step was to choose an ovine tissue where mRNA encoding for the EP₃R was expected to be abundantly expressed. The size of the product did not appear to be a problem because the expected size(s) of the product(s) were between 947-1037 and therefore, under the limit (1200 bp),

at least for abundant sequences.

Ovine studies

i) Accidental amplification of a mitochondrial sequence, when attempting to isolate the 3 cDNA ends of the ovine EP3R.

At high temperatures (55-60°C), a 585 bp band was often amplified from total RNA extracted from both the cortex and the external medulla of kidney. The product was shorter (585 bp) than the estimated expected size (872-1037 bp), and therefore controls omitting the adapter primer (P2) in the reaction, i.e. using the gene specific primer on its own (P3), were done and showed mispriming. However, because of the alternative splicing the expected sizes could be variable, and sequencing of the product from the renal medulla was done.

The comparison of this sequence with others stored in the Data Bank (Genbank/ EMBL), using the FASTA program of the GCG package, revealed very high homology (86.6%, Fig. 4.1) with the bovine mitochondrial cytochrome oxidase, subunit II (COII) (accession number M10544). This is the terminal enzyme of the electron transport chain of the inner membrane of mitochondria. It is involved in the generation of energy in the cell and therefore is very abundant in active cells (Anderson et al., 1982; Alberts et al., 1994b). Because the increase in the annealing temperature (up to 65°C), did not stop the mispriming to COXII cDNA, it was concluded that the PCR could not be optimised to obtain the EP₃ and a new gene specific primer was needed.

Figure 4.1

Sequence comparison of a fragment of the ovine (Scottish Greyface) mitochondrial cytochrome oxidase isolated here (in low case) with the corresponding bovine sequence (in upper case). The fragment isolated corresponds to positions 7560-7710 of the bovine mitochondrial genome (Anderson et al., 1982; accession numbers: JO1394 and VOO654).

```

1  acaatcttaaccattctaccagccattatcttaattatggattgctcttc 50
   ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
7560 ACAATCTGAACCATTCTGCCCGCCATCATCTTAATTCT.AATTGCTCTTC 7608
      .          .          .          .
51  catccttgcgaatcctatacataatggatgaaatcaacaacc.tctctc 99
   ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
7609 CTTCTTTACGAATTCTATACATAATAGATGAAATCAATAACCCATCTCTT 7658
      .          .          .          .
100 acagtgaagacc.tggggcatcaatgatactgaagctatgaatatacaga 148
   ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
7659 ACAGTAAAAACCATAGGACATCAGTGATACTGAAGCTATGAGTATACAGA 7708

149 at 150
    |
7709 TT 7710

```

ii) RACE products of the expected size

Using the RACE primers P3 and P2 and an annealing temperature of 55°C, a long band (between 955 and 734 bp) within the range of the expected size was observed on a single occasion. Work was not pursued with these primers because, as mentioned earlier (section 4.3.1i), higher temperatures favoured the amplification of the mitochondrial sequence rather than the EP₃R cDNA. Similarly, using a new gene specific primer (P4), an annealing temperature of 60°C for a long time (one minute) and an extension time of 1'30" in the PCR reaction, 3 faint bands of the expected size (700, 800 and 100 bp) were observed on a single occasion, within a smear going from 1200 to 200 bp. In other occasions, only the smear was visible. Using 1' extension time a smear and two shorter faint bands were observed of 700 bp and 400 bp. Controls for mispriming, using the gene specific primer on its own, did not produce any band or smear.

iii) RACE with a second set of RACE primers (P6 and 7)

The 1st set of RACE primers (P1 and 2), for the RT and the adapter primer of the PCR, were not identical to the original protocol (Frohman et al., 1988; Frohman, 1990) and we were unsure of their efficiency. Most authors were using the original sequences (P6 and 7), therefore they were tested but were also unsuccessful. No product was obtained using agarose gels or Southern blots (see next section) as detection methods. The total RNA used in these studies was extracted from the external medulla of the kidney, uterus, liver and adrenal gland.

4.3.2 Isolation of a cDNA fragment by RT-PCR

It was reasoned that the isolation and sequencing of a short fragment of the ovine EP₃ receptor by RT-PCR, would allow the design of ovine gene specific primers to improve the RACE method, as suggested by Walker and colleagues (1992). Knowing a stretch of the target sequence in the middle could theoretically allow the sequence of the full length cDNA to be obtained by using the RACE method in both directions, 3'end and 5'end (Walker et al., 1992; Olkkonen et al., 1994). It could be also useful to make probes for northern analysis to identify tissues expressing high levels of the receptor. Probes for Southern blotting could be used to screen the RACE products (specially in the case of no product or smears) for higher sensitivity and to identify the correct products before sequencing. Because of the alternative splicing, the RACE RT was expected to yield products of variable sizes, which may not be detectable as a band by normal gel electrophoresis, but as a "smear" (Taniguchi et al., 1993).

A bright band of the expected size (355 bp) was obtained from the external medulla of the ovine kidney using primers 4 and 5 at 55°C. The DNA from the PCR band (Plate 4.1) was isolated, re-amplified and sequenced, which allowed us to obtain a sequence of 267 nucleotides. This sequence was 98% homologous with the bovine EP₃ receptor cDNA (4 nucleotides different) and > 76% homologous with cDNAs of the rest of mammalian species published (Table 4.1 and Fig. 4.2). The deduced amino acid sequence only differed in 1 amino acid (1/89) with the bovine sequence (Fig. 4.3).

Plate 4.1

Ethidium bromide-stained agarose gel of RT-PCR product showing the isolation of a cDNA fragment of the ovine EP3 using bovine primers (lane 1). Lane 3: molecular weight markers and lane 2 is a blank.

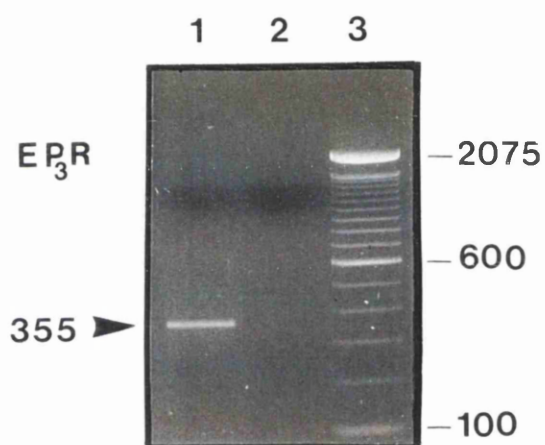


Table 4.1
Similarities between the ovine EP₃R cDNA sequence
and other species published.

species	nucleotide level (%)	aminoacid level (%)
bovine	98.5	98.9
human	87.6	87.6
rabbit	83.9	85.4
mouse	76.8	77.5
rat	76.4	79.8

ovine	GGC	GTA	TGG	CTG	GCG	GTG	CTC	GCC	TTC	GCC
bovine
humanGC
ratG	T.TG
mouseG	T.TG
rabbitGA
ovine	CTG	TTG	CCG	GTG	CTG	GGC	GTA	GGT	CAG	TAT
bovine
human	...	C..G	..CC
rat	..CTG	..C	..GC	..C
mouse	...	C..C	..GC	..C
rabbit	...	C.A	..TT	..G	..CC
ovine	ACT	ATC	CAG	TGG	CCC	GGG	ACT	TGG	TGC	TTC
bovine
human	..C	G..G
rat	..GC	G.GC	..G
mouse	..GC	G.GG	..C	..G
rabbit	..CG
ovine	ATT	AGC	ACT	GGG	CCT	GGG	GGC	AAC	GGG	ACT
bovineC
human	..CCGA
rat	..CCG	..C.A.	..G
mouse	..CCG	..C.A.	..A
rabbit	..CC	..A	..GAA.G
ovine	AAC	TCC	CGG	CAA	AAT	TGG	GGC	AAC	GTT	TTC
bovineC
human	..G.	..T	TC.	..T	..C	C..	...
rat	G..	..T	GC.	..GG	..G	CC.G.	..G	GC.
mouse	G..	..T	GC.	..GC	G.G	CC.G.	..G	GC.
rabbit	..G.	..T	TCA	..C	..C	C..	...
ovine	TTC	GCT	TCT	GAC	TTT	GCC	ATC	CTG	GGG	CTC
bovineC.
humanCC.	T..
rat	..T	..C	..C	..C.	..C	...	TGT	..A	..C	T.G
mouse	..T	..C	..C	..C.	..C	...	TG.	T..	..C	T.G
rabbitC	..C	AC.	T..C	...
ovine	TCG	GCG	CTG	GTG	GTC	ACC	TTC	GCC	TGC	AAC
bovine
human	..T.	ACAT	T..
rat	CT.	..TT
mouse	CT.	..TGT
rabbit	..T.CC	A..
ovine	CTG	GCC	ACC	ATT	AAG	GCC	CTG	GTG	TCC	CGC
bovine
human
rat	T..	..GC	..AA
mouseGC	..A
rabbitT
ovine	TGC	AGG	GCC	AAG	GCG	ACG	GCG	TCG	CAG	
bovineC	
human	...	C..CA	..T	...	
rat	..CA	..C	G.C	..C	
mouse	..T	C..A	..C	G.C	..C	
rabbit	...	C..	..A	G.A	..A	..A	...	

Figure 4.2

Alignment of nucleotides, comparing the partial codings of the isolated ovine EP₃R cDNA with other species. Dots indicate identical nucleotides. The position of the partial sequences shown correspond to nucleotides 537-801 of the bovine sequence (Namba et al., 1993).

Ovine sequence has Genbank Accession number U37148.

Figure 4.3

Alignment of deduced aminoacids, comparing the ovine EP₃R with other species. Dots indicate identical aminoacids. The position of the partial sequences shown correspond to aminoacids 179-267 of the bovine sequence (Namba et al., 1993).

ovine	G	V	W	L	A	V	L	A	F	A
bovine
human	S
rat	S
mouse
rabbit.
ovine	L	L	P	V	L	G	V	G	Q	Y
bovine
human
rat	R	.
mouse	R	.
rabbit
ovine	T	I	Q	W	P	G	T	W	C	F
bovine
human	.	V
rat	S	V
mouse	S	V
rabbit	.	I
ovine	I	S	T	G	P	G	G	N	G	T
bovine
human	R
rat	A	.	.	E	.
mouse	A	.	.	E	.
rabbit	R	.	D	.	.	.
ovine	N	S	R	Q	N	W	G	N	V	F
bovine
human	S	.	S	H	L	.
rat	D	.	A	R	E	P	.	S	V	A
mouse	D	P	A	R	E	P	.	S	V	A
rabbit	S	.	S	H	L	.
ovine	F	A	S	D	F	A	I	L	G	L
bovine	.	.	.	A
human	.	.	.	A	.	.	F	.	.	.
rat	.	.	.	A	.	.	C	.	.	.
mouse	.	.	.	A	.	.	C	.	.	.
rabbit	.	.	.	T	.	.	F	.	.	.
ovine	S	A	L	V	V	T	F	A	C	N
bovine
human	L	.	.	T	.	.	.	S	.	.
rat	L
mouse	L
rabbit	L	.	.	A	I
ovine	L	A	T	I	K	A	L	V	S	R
bovine
human
rat
mouse
rabbit
ovine	C	R	A	K	A	T	A	S	Q	
bovine
human
rat	A	.	.	.
mouse	A	V	.
rabbit	A	.	.

4.3.3 Studies of the mRNA expression of the ovine EP₃ receptor by RT-PCR and Southern blotting.

Initially, northern analysis was carried out, using a probe prepared from the PCR product (primers 4 and 5) to study mRNA expression in several tissues (cervix, uterus and different segments of the kidney), but no signal was observed in any of the membranes or the tissues tested. Expression has been found using northern analysis in the uterus and the kidney of other species by others and therefore it was thought unlikely that the sheep did not express the receptor. Low levels were suspected that may be undetectable by northern analysis. It was decided that RT-PCR, being 1000-10,000 times more sensitive than northern blotting (O'Shaughnessy, personal communication; Liu et al., 1996), may be more suitable to detect expression of this receptor in ovine tissues.

4.3.3i DNase I incubations to eliminate genomic DNA contamination

Initial studies looking at EP₃R expression by RT-PCT appeared to show expression in all tissues tested. Controls missing the RTase from the RT and treatments with DNase I confirmed the suspicion that DNA contamination of an intronless fragment was creating a problem of false positives. But, following DNase incubations, results for EP₃R mRNA expression turned negative in all tissues tested.

A series of studies followed, aiming at the investigation of whether or not the negative results were artefacts due to RNA degradation during the DNase incubations (chapter II). It was found that some RT buffers had RNase activity which degraded RNA during the DNase incubation (Plate 4.2A). In subsequent studies, the use of RNase-free Tris (Sigma), Mg filtered in a 0.2 mm Millipore and the control of the pH (6.8-7.2) were enough to prevent RNA degradation (Plate 4.2B).

Plate 4.2

A) Degradation of RNA during the first DNase I incubations due to RNase activity in common RT buffers.

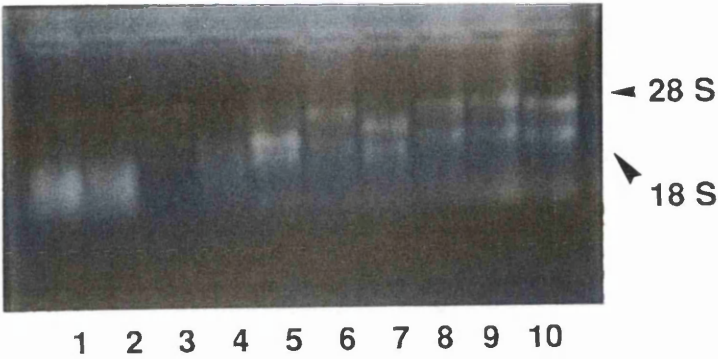
RNA samples run in an ordinary 1% agarose gel. In lanes 1 to 4 RNAs are completely degraded (the ribosomal bands 18S and 28 S cannot be seen) after incubation with several buffers. In lanes 5 to 7, RNAs are intact when mixed with the buffers but omitting the incubations. Lanes 8 to 10 are RNAs without buffers or incubations and they are clearly intact.

B) Undegraded RNAs following DNase I incubations under optimal conditions.

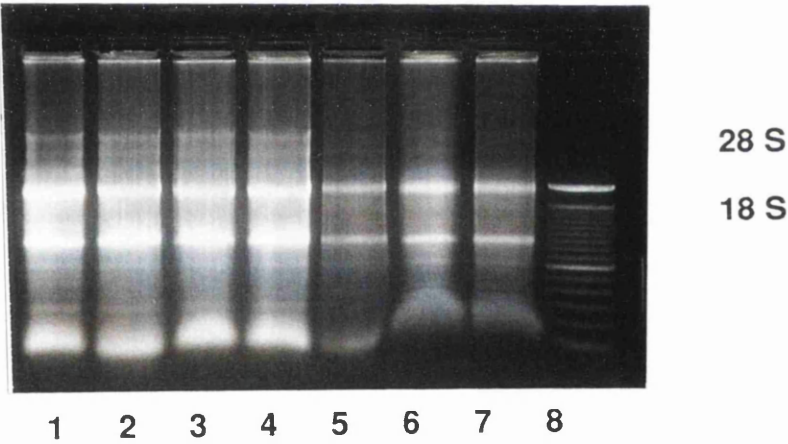
Lanes 1 to 7: RNA samples run in agarose gel, they are intact (shown by clear ribosomal bands; arrows) following DNase incubations under optimal conditions

Lane 8: DNA molecular weight marker for size comparison.

A)



B)



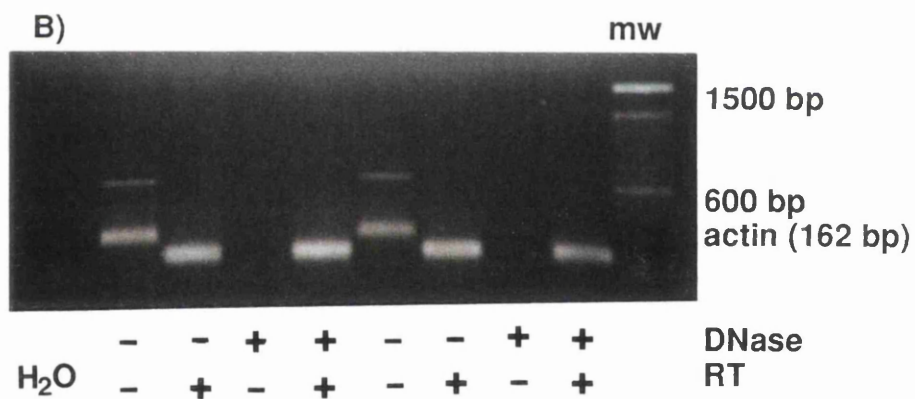
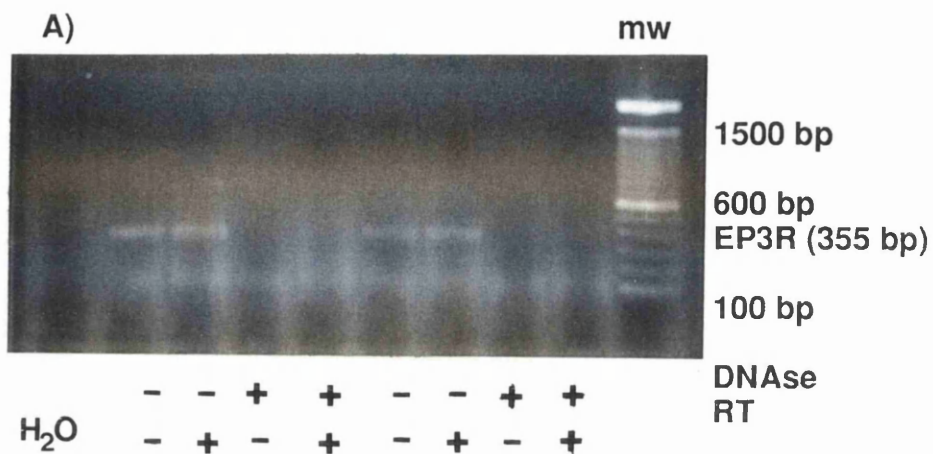
Despite the optimization of the DNase incubations, which did not degrade RNA, mRNA expression could not be demonstrated in any of the tissues tested, as illustrated in the next plate (Plate 4.3). To exclude the possibility that ethidium bromide was not sensitive enough to detect the EP₃ signal Southern blots were carried out, with the same results. Another possibility was that the ovine tissues investigated here (kidney, uterus and cervix uteri) did not express mRNA encoding for the EP₃R, unlike other species. Other ovine tissues were investigated as alternative sources, bovine adrenal medulla, ovine adrenal medulla, ovine ovary and ovine skin with subcutaneous tissue, but results remained negative.

Plate 4.3

False negative results

A) Ethidium bromide-stained agarose gel of RT-PCR products showing lack of mRNA expression of the ovine EP₃ receptor cDNA. Amplification of template following an incubation with or without DNase and reverse transcriptase (RT+/-). The molecular weight marker was 100 bp ladder (Gibco). The presence of a band in the RT-/DNase- indicates contamination by genomic DNA. The absence of a band in the DNase+/RT+ represents negative result for the expression of mRNA, which were initially obtained.

B) Amplification of ovine β -actin used as a positive control to demonstrate the integrity of the RNA and that the RT reaction apparently worked.



The successful amplification of actin cDNA following the digestion with DNase I showed that RNA was not degraded and nor was the RT affected by the DNase incubations. The amplification of the EP₃R cDNA from genomic DNA (i.e. without DNase incubations) showed that primers and the PCR conditions were working.

4.3.3ii Demonstration of mRNA expression of the ovine EP₃R in the cervix and other tissues

If there was a technical problem causing false negatives, it was "pre-PCR", either RNA degradation or failure of the RT, since the PCR primers appear to work, being able to amplify genomic DNA. The problem only affected some target sequences (ovine EP₃R cDNA) whereas it did not affect the amplification of other abundant sequences, mainly steroidogenic enzymes by other people in the laboratory or the ovine β -actin by the author.

It was discovered that by mistake a batch of low concentration of dNTPs had been used in the laboratory for several months. It was thought that this could have affected the reverse transcription of fragments longer than ovine β -actin or sequences of low copy numbers. A number of tissues were tested with the corresponding RT minus and RNase free DNase I minus controls, correcting the dNTP concentration and positives were found for mRNA expression in the following ovine tissues: kidney external medulla, liver, uterine body, oestrous cervix, luteal cervix and skin (Plates 4.4 and 4.5). Therefore all the tissues tested were positive, with the exception of prepartum cervix (within 1-2 days of labour). But using Southern blotting to increase sensitivity, cDNA was shown to be present in these samples too (Plate 4.5). The RT-minus controls were negatives, demonstrating that DNase I still worked well and the product in the DNase+/RT+ reaction was in fact mRNA and not genomic DNA.

Plate 4.4

Ethidium bromide-stained agarose gel of RT-PCR products showing expression of the ovine EP3 receptor mRNA in the liver (lane 1). Using this method, expression was also found in the kidney, uterus, adrenal gland and skin. False negative results in the previous plate were due to suboptimal conditions in the RT reaction.

Expression of the ovine EP3 receptor

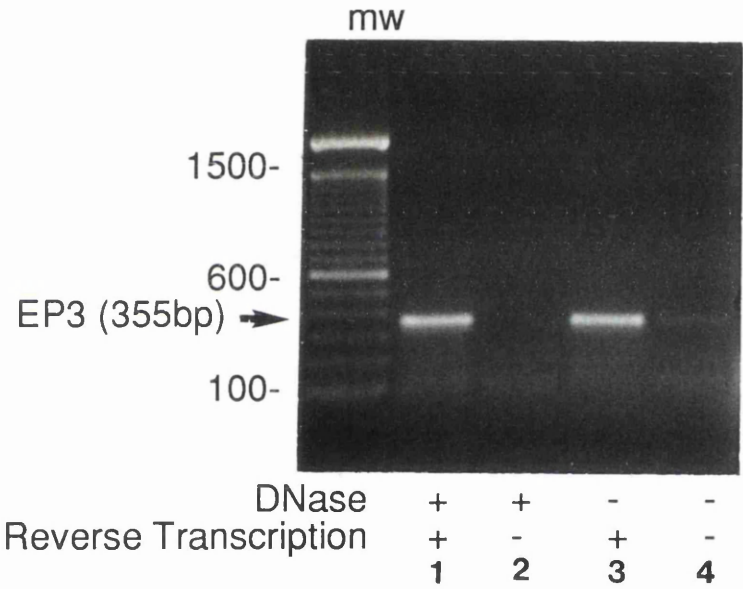
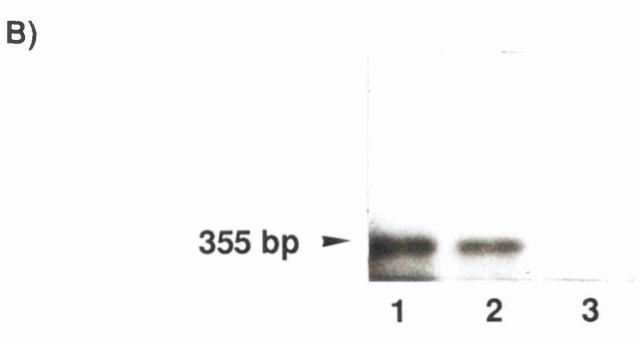
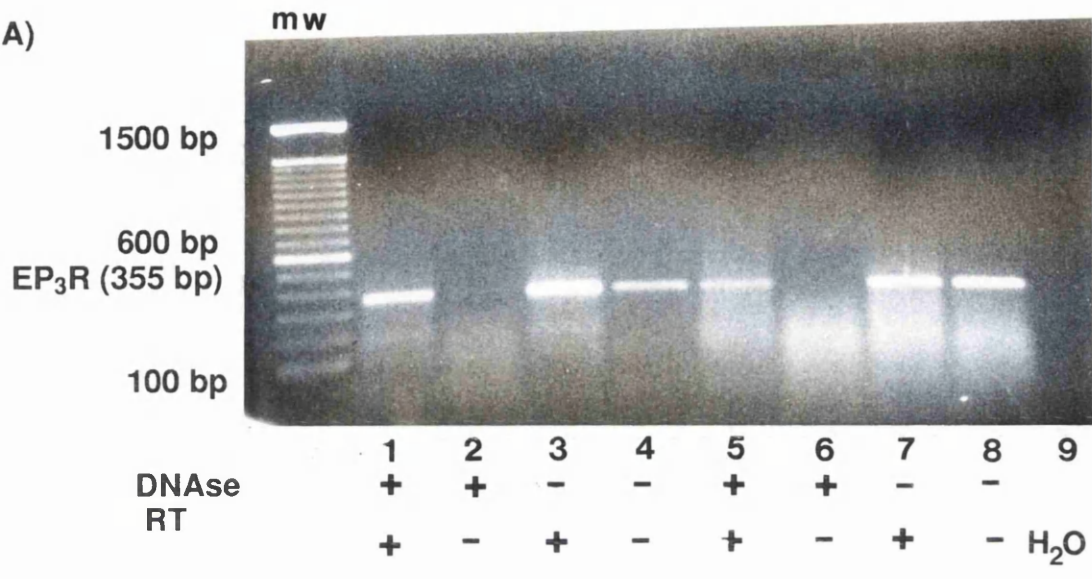


Plate 4.5

Ovine EP3 receptor cDNA amplified from cervix during the oestrus cycle. The molecular weight marker (mw).

A) amplification of EP3R cDNA from cervix at oestrus (lane 1) and dioestrus (lane 5). The absence of genomic DNA contamination is shown in lanes 2 and 6. Lanes 3, 4, 7 and 8, show the presence of genomic DNA when DNase I is omitted. Lane 9 is a blank.

B) Southern blot analysis of RT-PCR products showing expression of the EP3 receptor in the ovine cervix at peripartum (lane 1) and oestrus (lane 2). Lane 3 is a blank.



4.3.3iii Attempts to quantify mRNA expression by RT-PCR

Work was done to reduce the sources of variation which might affect the RT-PCR technique. The absence of RNA degradation was confirmed by visualisation of the ribosomal bands in agarose gels and controlled for by simultaneous amplification of a suitable housekeeping gene. The latter also controlled for the variability in performance of the RT. The PCR was also optimised to reduce the strong primer dimer observed with the initial conditions and to improve the consistency of the signal (see materials and methods for more details).

When reducing the cycle numbers in the PCR of the EP₃R (P4 and 5), the bands were not clearly visible in agarose gels or after autoradiography, which made cutting the bands for scintillation difficult. Probably because of low levels of the product and also because the RT-PCR was not completely optimized, yielding primer dimmer on some occasions. Because the usual procedures for optimization were already attempted in every step of the reaction: RNA extraction, RT and PCR (Innis and Gelfand, 1990; Kid and Ruano, 1995; Roux, 1995), this was not pursued any further. Despite these problem, the actin and EP₃R cDNA bands were cut and counts were read, but those from the EP₃R reaction did not show exponential increases and therefore quantification was not possible.

4. 4 Discussion

4.4.1 Isolation of a cDNA fragment by RT-PCR and confirmation of its identity

This study reports the successful isolation of a fragment of ovine cDNA encoding for the EP₃R by RT-PCR, from RNA extracted from the external medulla of the kidney and using relatively long (25 bp) bovine primers. This fragment is part of the functional EP₃R mRNA, which is supported by the following evidence: (1) the high degree of similarity between its sequence and that published for the bovine, and other mammalian species, (2) the detection of mRNA expression following DNase I incubations, (3) the similar pattern of mRNA expression found in sheep tissues when compared with other species. These points will be expanded in the next paragraphs.

Receptors and other genes usually exhibit high degree of conservation through evolution in vertebrates, and in most cases the aminoacid and nucleotide sequences are conserved across species (Watson and Girdleston, 1995; Sealfon, 1995; Anderson et al., 1996). We have observed here an homology ranging from 76.4 to 98.5% between the ovine EP₃R cDNA and other species of mammals published, which is comparable to homologies found in ovine studies of other GPCRs and several cytokines (Stewart et al., 1993; O'Brien, 1994; Reppert et al., 1994). The very high homology observed between the bovine and ovine EP₃R cDNAs (98.5%) is not surprising, due to the closeness of these species phylogenetically and the conservation of prostanoid receptors within mammals (Toh et al., 1995). Studies on evolutionary genetics, have shown that goat and sheep, diverged from the cow relatively recently, about 7-10 million years ago. Other mammals (pigs, rodents, humans), whose sequences are often used to amplify ruminant sequences diverged 60-100

million years ago (Irwin et al., 1991; Hiendleder et al., 1991).

Some authors however, have the opinion that the identity of a receptor cannot be based on high homology with other species only, due to the existence of pseudogenes (Sealfon, 1995). Pseudogenes can have very high homology (70-90%) with the corresponding gene. They are common in multigene families, and several ones have been found among GPCRS, including one of the prostaglandin E₂ receptors in humans (the EP₄R pseudogene) (Ahuja et al., 1992; Sealfon, 1995; Foord et al., 1996). The cDNA fragment isolated here is unlikely to be a pseudogene because: 1) mRNA expression was found, and although some pseudogenes are transcribed, most of them, including the EP₄R, are not expressed (Foord et al., 1996). 2) Furthermore, Southern blots have only been able to detect a single copy of the EP₃R gene in other species, and therefore there are no reported pseudogenes of the EP₃R gene so far (An et al., 1994).

To further support the identity of the isolated cDNA, it was important to confirm that this mRNA was expressed and that its localisation in tissues was similar to other species. In order to do this, DNase I incubations of the RNA were required because it was found in these studies, that the ovine gene encoding the EP₃R between the second intracellular loop and the beginning of the VI TM domain is intronless, as reported in other species too (Regan et al., 1994a; An et al., 1994; Breyer et al., 1994; Tarng and Breyer, 1995). When intronless fragments are amplified, contaminating DNA is non-differentiable from cDNA which can create false positives, as seen here initially. Following DNase incubations, mRNA distribution was shown in several ovine tissues (liver, kidney, uterus, adrenal medulla and skin). This pattern of mRNA expression is consistent with that from other species (man, rat, rabbit and mouse: Namba et al., 1993; Takeuchi et al., 1993; Sugimoto et al., 1993; Adam et al., 1994; Breyer et al., 1994; Negishi

et al., 1995), which further confirms the identity of the cDNA isolated as the ovine EP₃R homologue. Although the ultimate confirmation involves more complex experiments of transfection of cells with the cloned cDNA to demonstrate the appropriate ligand binding and/or signal transduction coupling (Ushikubi et al., 1995; Sealfon, 1995).

Summarising, some publications claim to have isolated or cloned the homologue of certain cDNA in a new species, based on sequence homology only (Radford et al., 1991; O'Brien et al., 1994; Sealfon, 1995). Here, apart from this evidence, mRNA expression is also demonstrated to further support that the cDNA isolated is in fact part of the functional ovine EP₃R cDNA. This sort of evidence has also been used by others with another GPCR (Stewart et al., 1993). This work therefore has provided new reagents for the study of the EP₃R, "universal primers" and a cDNA sequence that may be used to clone a full ovine EP₃R or to study the receptor in other species of veterinary interest. It would be interesting to see how "universal" the primers are, very high sequence homology and antibody cross reactivity may be expected between the ovine and caprine EP₃R and high homology within Ungulates in general and other mammals. An interesting challenge would be now the cloning of cDNA from non mammalian species (see more in General Discussion: chapter VI).

4.4.2 The experience gained using RACE for the isolation of the 3'end of the ovine EP₃R cDNAs

In this work, we were unable to isolate any ovine cDNA using RACE. Restrictive parameters for the success of this method, like with other PCR methods, are whether the sequence of the protein is known, the GC/AT ratio of the target sequence, the abundance of the mRNA message in the selected source and the size of the cloned cDNA. But obviously to amplify cDNA with a single gene specific primer is harder than using two primers, and mispriming is more likely to occur (Sambrook et al., 1989; Zullo et al., 1993; Simonsen and Lodish, 1994; Frohman, 1994).

During these studies, RACE has not been used by others to amplify the 3'ends of the EP₃R on any new species, but there are reports of the successful amplification of this and other prostanoid receptors, knowing part of the gene sequence (Tarng and Breyer, 1995; Ogawa et al., 1995; Båtshake et al., 1995). Similarly, there are several reports on the use of RACE to obtain species homologues or members of a gene family. RACE can work in those cases, but other parameters need to be favourable (reviewed by Frohman, 1994). These works by others suggest that the isolation or cloning of ovine EP₃R cDNAs by RACE, may also be feasible although some knowledge of the sequence may be helpful. Not knowing the sequence and having to use a single primer may reduce the specificity and efficiency of the PCR considerably. In RACE, the gene-specific primer could not be located too far apart from the C-terminal, or the product may be too long to be amplified efficiently and not knowing the target sequence was a further limiting factor in the design of this primer to avoid mismatches (see Materials and Methods about the requirements at the 3 prime end).

The importance of the gene specific primer in relation to the abundance of the message is highlighted by the amplification of a

mitochondrial sequence, due to mispriming. Cytochrome oxidase, subunit II (COX II), is encoded by the mitochondrial genome, which is intronless. The mRNA encoding for COX II is likely to be more abundantly expressed in cells than the EP₃R, because the former is part of the respiratory chain that generates energy in the cell (Alberts et al., 1994b). In addition, mitochondrial DNA (mtDNA) is 1000 times more abundant than nuclear DNA in somatic cells (Zullo et al., 1993; Hecht, 1990), which therefore constitute most of the DNA contaminant in RNA samples. The coamplification of mitochondrial DNA and mRNA can occur if there is homology with a single primer, because of much higher abundance in the cell when compared to any nuclear sequence (Zullo et al., 1993). Mispriming to mitochondrial sequences is a common artefact of nuclear DNA amplification and also of cDNA libraries construction (Zullo et al., 1993; Wenger and Gassmann, 1995). Since the mitochondrial genome is not very large (about 16 kilobases) and it has been completely sequenced in many species now (Hecht et al., 1990; Broad and Hill, 1994); mispriming can be prevented by checking the primers against the gene bank for the absence of homology with mtDNA (Kidd and Ruano, 1995).

To increase sensitivity and specificity, the original RACE protocol has incorporated recently the use of nested primers, either for one primer (the gene specific primer) or both (Frohman, 1994). These approaches were not used here, but they may increase the chances to clone isoforms of the ovine EP₃R by RACE. The use of nested homologous primers could have contributed to the success in the amplification of 3'end of other prostanoid receptors by others (Ogawa et al., 1995; Båtshake et al., 1995). The partial ovine cDNA sequence obtained here, whose accuracy can be confirmed, may be used to design one or two nested ovine gene-specific primer(s).

To isolate cDNA encoding for the ovine EP₃R receptor, it was crucial to choose a rich source, logically the higher the concentration of this sequence in the starting cDNA, the easier the amplification and isolation. Recent estimates of the number of mRNA copies per cell of the EP receptors suggest that they fall within the category of "very rare messages", since there are only 10 or <1 copies per cell (Sambroock et al., 1989; Tsai et al., 1996; Blaschke et al., 1996). Furthermore, when RNA is extracted from tissues, as in our case, these consist of several cell types that may or may not express the target gene (Sambroock et al., 1989; O'Shaughnessy, pers. comm.; Simonsen and Lodish, 1994). A fully optimised RT is important to obtain enough first strands of the target sequence for subsequent amplification, but this is even more critical when the message is rare. Here, after correcting the RT conditions, based on the normal RT-PCR studies, several products were obtained, but the work was not pursued any further with the identification of these products by sequencing or Southern blotting.

At the beginning of these studies high copy numbers per cell were expected in tissues selected as rich sources, because others reported high levels of expression by northern analysis which is 1000-10000 times less sensitive than PCR, which suggested that the message was amplifiable by PCR techniques. Later, RT-PCR was used aiming at the identification of alternative rich sources of EP₃R mRNA and possible sublocalization within the kidney. But low levels were detected in all the tissues tested (kidney external medulla, kidney cortex, kidney internal medulla, uterus, adrenal gland, skin and liver).

A recent abstract suggests that the large luteal cells of a 10 day corpus luteum, which was not tested here, may be a good source to clone the ovine EP₃R (Pierce et al., 1995b), but this information has not been fully published and quantitative results in the cow at a similar stage (day 8) show

very low expression in *corpus luteum* (Tsai et al., 1996). Until further clarification of the level of expression of these receptors is available, it appears that cell isolation and enrichment of the mRNA message may be necessary for cloning by PCR techniques. This sort of approach is common place when cloning local mediators like cytokines (Argyle, 1996). Recent studies are suggesting that the EP₃R may be upregulated in immature animals and that upregulation may be inducible with indomethacine, a prostaglandin synthesis inhibitor (Hashimoto et al., 1991; Bonilla and Jiang, 1996). Obviously the gene characterisation will give more clues as to how the expression can be upregulated, to enrich the source for cloning.

In conclusion even those tissues considered as rich sources here and by other authors (kidney, uterus, liver, adrenal medulla and *corpus luteum*) appear to express low levels of EP₃R mRNA, although this needs further clarification. At the moment, it appears that these tissues may be suitable sources for cloning with RACE but the task is technically demanding. The use of kits may be justified to minimise problems of suboptimal reactions and the yield of the RACE may be increased by using cell isolation and enrichment of the mRNA message, short cDNA end size and homologous nested primers.

4.4.3 expression studies

i) Quantitation

In this work it was intended to quantify EP₃R mRNA expression, to compare levels between positive controls and the cervix to investigate a possible role in this tissue. If expression could be found in the cervix, levels could be then compared between different reproductive stages (oestrus, early luteal phase and peripartum).

It is now known that quantitative mRNA studies for the prostanoid receptors are difficult to do by northern blotting and they are best done by RT-PCR (Blaschke et al., 1996). Here the RT-PCR assay was able to detect expression in those tissues but the low efficiency of the PCR, despite efforts of optimisation, did not allow quantification. For the isolation of a certain cDNA, primers should be specific, but for semiquantitative assays it is very important that they are efficient in the amplification as well. If the PCR works efficiently, during the exponential phase the amount of amplicons is almost doubling at each cycle, which allows calculations of the number of initial copies from measurements of the products (Dieffenbach et al., 1993; Ferre, 1992).

When mismatched primers are used, spurious products are produced that may compete for the primers and other reagents and do not allow doubling of the desired amplicons on each cycle. The primers used here were relatively long (25 bp). Primers can be as long as 35 bp and their use is recommended in cross-species PCRs, because each nucleotide increases the specificity four times. But it is difficult to calculate the annealing temperature when using long or mismatches primers, which makes PCR optimisation more difficult (Dieffenbach et al., 1993; Roux et al., 1995). In addition, although DNase incubations did not grossly degrade RNA, it probably contributed to variability in the amplifications.

In conclusion, assuming that the reactions are optimised, primer design is crucial for the success of PCR reactions. The balance should be kept between two goals: specificity and efficiency of the amplification (reviewed by Dieffenbach et al., 1993). Specificity is the main goal for the isolation of new cDNA sequences, this is to amplify the desired sequence and not others. But to establish a semiquantitative assay, the efficiency should be maximal to obtain exponential amplification. The primers used

here were not very efficient and quantification was not possible. But it is anticipated that this may be possible using a new set of primers, preferably ovine, shorter (24 to 18) and located in different exons.

ii) EP₃R mRNA expression in the ovine cervix

Our studies have shown EP₃R mRNA expression in the cervix for the first time, suggesting a new function in this tissue. Neither the presence of the mRNA nor the protein have been reported before in the cervix of any species. A number of studies suggest a paracrine role for PGE₂ in the cervix, based on local synthesis, binding sites, second messenger studies and the clinical effect of PGE₂ and analogues in the cervix (Ellwood et al., 1981; Norström et al., 1983; Rao, 1990; Uldbjerg and Ulmsten, 1990; Owiny et al., 1990; Bygdeman, 1992). None of these studies show, however, which EP (EP₁-EP₄) receptor(s) are involved.

The RT-PCR studies reported here are not quantitative but the level of mRNA expression in the cervix appears to be very low, specially in the samples of late pregnancy where mRNA was only detectable by Southern blotting. It is important to consider the possibility of further sublocalisation within the cervix. Inflammatory responses are localised mainly in the endocervix (see chapter III), where higher levels of the EP₃R may be present. Similarly, PGE binding site studies have shown a low density in the whole cervix (Rao et al., 1990) which may be due to low receptor numbers in the fibromuscular wall.

The EP₃R mRNA levels were low in the cervix, irrespective of the stage studied. Because no upregulation of mRNA was obvious in oestrous or in periparturient sheep, it is tempting to speculate that oestrogen does not appear to upregulate the mRNA expression of this receptor. Logically proper quantification of the EP₃R mRNA and its protein are required to

clarify if the number of EP₃Rs is highly regulated in the first place and if this involves mRNA regulation. The elucidation of the gene structure, particularly at the promoter region, can also give clues of the presence of regulatory elements for steroids, prostaglandins or other inflammatory mediators, as possible candidates. Little is known about the regulation of the genes of prostanoid receptors, but proinflammatory response motifs are also present in the TR and EP₄R genes (Nürsing et al., 1993; Foord et al., 1996). This type of regulation would be consistent with the inflammatory theory of the onset of labour and cervical ripening. Other workers, as commented before in this discussion have found evidence of the regulation of the EP receptors, by the synthesis of PGE₂ (Hashimoto et al., 1991; Zeng and Goetzl., 1995).

CHAPTER 5

ULTRASOUND IMAGING OF THE OVINE CERVIX DURING THE OESTROUS CYCLE

5.1 INTRODUCTION

5.1.1 Basic ultrasound principles

This section draws together information from the following sources: Ginther, 1986; Taverne and Willemse, 1989; Paterson, 1990; Griffin and Ginther, 1992; Boyd and Omran, 1991; Nyland et al., 1995.

Ultrasonic imaging utilises sound waves of higher frequencies (2-10 MHz) than the upper range of the human hearing (20 KHz) to produce images of tissues and internal organs. The technology is based and follows the physics of the reflection that waves experience when they hit the boundary between two surfaces of different acoustic impedance, this phenomenon is known as "the echo principle". In practical terms, due to differences in composition and density, tissues vary in their ability to reflect ultrasound waves. Echoes from tissues are obtained, exploiting the properties of piezoelectric crystals, which permits the scanning with a transducer that is both the generator of sound waves and the detector of echoes returning from the tissues. Sound waves are generated in the transducer by the vibration of the piezoelectric crystals when they are exposed to a high-voltage electric current. Conversely, these crystals can also convert the returning sound waves from tissues into an electric current that can be measured and displayed as an image in the scanner screen.

Ultrasound scanners used in veterinary reproductive research are B-mode (brightness modality) real time scanners, they produce a two dimensional display of pixels that represents a real time anatomical image. Real time refers to the ability to display image movement as it occurs, which is often recorded using videotapes. The brightness of the pixels is proportional to the amplitude of the reflected echoes returning to the transducer. A grey scale (black to white) is also assigned to each pixel,

corresponding to the amplitude or strength of the returning echo. Very low intensity echoes are displayed as black (anechoic), mid range echoes as varying shades of grey (echogenic), and high-intensity echoes as white (hyperechogenic). Tissues are therefore defined by certain ultrasonic characteristics (echotexture or echogenicity), which reflect the histological characteristics of the tissue.

The ability to image structures lying closely as separate entities depends on the resolution of the ultrasound beam which has two components, axial and lateral. Axial resolution is the ability to identify closely lying structures along the propagated direction of the sound wave (the beam axis). This is determined by the length of the wave, which assuming a constant velocity in tissues, is inversely proportional to the frequency that the transducer generates. Normally, each ultrasound transducer emits sound waves of a fixed single frequency and the ultrasonographer therefore has to select higher frequencies to achieve short wave lengths and better resolution. Lateral resolution refers to the ability to resolve adjacent points perpendicular to the axis of the sound beam. This is dependent of the ultrasound beam width, the size of the transducer face and the distance between the tissue and the transducer. Modern transducers have the ability to focus to ensure good lateral resolution. Velocity of ultrasound waves is almost constant in soft tissues (1540 m/sec.) but is lower in gas (331 m/sec.) and higher in bone (4080 m/sec.), these differences produce various artefacts that will be discussed later.

Penetration is another important issue when selecting the transducer and the scanning technique to be used. Ultrasound waves progressively weaken, losing amplitude (attenuation) as they travel from the transducer through tissues, mainly due to absorption, reflection and scattering of the sound beam. Lower frequencies suffer less attenuation than higher frequencies and therefore are used when deep penetration is required. As

explained earlier, low frequency transducers produce poor resolution, so a compromise between penetration and resolution has to be found when choosing the transducer. Other factors that contribute to attenuation in practical terms are the size of the animal and the number of tissues between the transducer and the tissue to be imaged. Attenuation may be minimised in large animals by introducing the probe inside body cavities rather than using a transabdominal approach. For example, transrectal scanning reduces the distance travelled by the echoes and has been very useful in reproductive studies of large animals, permitting the use of high frequency transducers to obtain high resolution images with adequate penetration.

5.1.2 The use of B- mode ultrasonography in the reproductive tract of the ewe in comparison with other animal species.

The use of real time B-mode, grey scale ultrasound with transrectal linear probes, in veterinary reproductive studies started in 1980 in mares and has been used extensively since , both in the field and for research purposes (reviewed by Ginther, 1986; Griffin and Ginther, 1992). The technology was adapted from human obstetrics, which at the time employed low frequency transducers (3.5 MHz) for transabdominal use (reviewed by Paterson, 1990). Later more robust transducers became available which were suitable for transrectal scanning of mares and cows, but the technology was still unsuitable due to the low frequency of the transducers that produced poor resolution and unwanted excessive penetration (Omran, 1989; Boyd and Omran, 1991). Higher frequency transducers (5 and 7.5 MHz) became available later due to veterinary demand for ovarian work in large animals (Ginther, 1986; Boyd and Omran, 1991).

Using high frequency transducers transrectally, it is possible to obtain very detailed information on the reproductive tract and the ovarian function in large species. The large size of the rectum in these species, allows handheld

transrectal scanning, positioning the transducer over the reproductive organs in the longitudinal and transverse axis (Omran, 1989). In large animals three linear transducers for endorectal use (3.5 MHz, 5 MHz and 7.5 MHz) are commonly employed in the gynaecological examination of the mare and the cow (Boyd and Omran, 1991; Griffin and Ginther, 1992). Other animals (e.g. llamas and deer) in which rectal palpation is possible can also benefit from the experience gained in mares and cattle. Applications of this technique include; examination of ovarian structures and determination of the stage of the oestrous cycle, investigation of uterine or ovarian pathology, improvement of AI technique, early pregnancy diagnosis and studies of the embryo/foetal development (reviewed by Ginther, 1986; Omran, 1989; Taverne and Willemse, 1989; Boyd and Omran, 1991; Griffin and Ginther, 1992; Beal et al., 1989).

In sheep, the applications of B-mode ultrasonography in the field during the 1980s and early 1990s, had been limited to pregnancy diagnosis and the prediction of foetal numbers. This was normally carried out using mainly the transabdominal technique and a 5 MHz sector transducer (Taverne and Willemse, 1989). In recent years, the endorectal technique has also been employed for this purpose, but the technique has not been established as in large animals (Kähn, 1994). In small ruminants, research has been performed on the determination of the gestational age using the transabdominal technique for embryo/fetometry (reviewed by Griffin and Ginther, 1992; Picazo et al., 1991). Other applications of the technology, (e. g. the anatomical study of the non pregnant tract, assessment of ovarian activity and the study of genital disorders) have not been exploited in small ruminants, as much as in large animals (Griffin and Ginther, 1992; Kähn, 1994).

The first application of the ultrasonographic imaging of the ovine ovaries was the monitoring of the ovarian response to eCG in Booroola sheep, utilising sector probes (3.5 and 5 MHz) and the transabdominal technique (Bor et al., 1992). Other authors reported the transcutaneous use of a 7.5 MHz sector transducer to scan ovine ovaries autotransplanted to a site under the skin in the neck. In these studies follicular dynamics were described, but sonograms were not shown (Campbell et al., 1991a; Campbell et al., 1991b; Campbell et al., 1994).

The transrectal imaging of the non-pregnant reproductive organs in small ruminants requires higher resolution than in large animals (Buckrell, 1988). In addition, the small diameter of the rectum does not allow internal manipulation of the tract by hand or the orientation of the transducer in the transverse plane, making the imaging of the various organs more difficult (Kähn, 1994). Because of these difficulties, suitable endorectal probes for small animals were not available until the mid 1990s when their use began to be documented (Kähn, 1994). Early studies in small ruminants, using a 5 MHz transrectal transducer designed for mares, concluded that the follicles could not be identified (Buckrell, 1988). Similarly, anatomical studies of the ovine cervical canal to develop a transcervical AI technique had been unsuccessful, although the authors did not give any details about the equipment employed (Halbert et al., 1990a).

The type of transrectal ovarian and early foetal studies that appeared in the 1980's in large animals has just begun in small ruminants, this is due to the availability of small rectal probes and better ultrasound equipment in veterinary research institutions. The first practical application of transrectal ultrasonography in the small ruminant ovary was the identification of the ovarian response to superovulatory treatments (Dorn, 1989). Ultrasonographic images of normal ovaries of small ruminants were documented in a water bath and by transrectal examination, but it was

pointed out that the smaller follicles could not be resolved with the technology available at the time (Kähn, 1994). Later, two publications described follicular dynamics in the ovary of the ewe, using transrectal scanning with a 7.5 MHz linear transducer designed for the study of the prostate in humans (Schrack et al., 1993; Ravindra et al., 1994). Similarly, the examination of the viability of the embryo has been achieved in sheep using a transrectal probe of 7.5 MHz (Kaulfuß et al., 1994).

New developments in ultrasound transducers has led to the introduction of specialised probes that have been designed to examine inside body cavities and to allow positioning of biopsy instruments. Intracavitary ultrasound imaging (transvaginal and endorectal) has benefited recently with the design of transducers of low radius curved array (microconvex transducers). These probes have a small scanning face with series of crystals eccentrically oriented along the longitudinal axis increasing the field of view without compromising high resolution (Thaler and Manor, 1990; Gelly, 1992). In human obstetrics this has been used for various purposes, including the monitoring of cervical ripening (Andersen and Ansbacher, 1991). These transducers have also facilitated aspiration of ova, follicular fluids and foetal fluids in cattle (Scott et al., 1994). Transducers for human use now have auto-focus and other facilities that improve lateral resolution.

5.1.3. The Endorectal technique for the gynaecological examination in ruminants

Equipment used for endorectal scanning

Both linear and sector scanners can be used endorectally in reproductive studies, but linear transducers are usually preferred in routine work for large animals because they produce a rectangular image that is easier to interpret, without distortion of the sonogram in the near field. In general, linear transducers have a longer durability and are more economic (Boyd and Omran, 1991). In sheep, linear transducers are used endorectally because they are more ergonomic than sector (Kähn, 1994). The disadvantage of the linear transducers is the large size of the scanning surface which requires a large surface of contact with the body, this is more of a problem for small animals for two reasons: 1) cross sections obviously cannot be obtained and 2) lateral resolution is poor unless the transducer is focused (Paterson, 1990). It has been tackled in human urology and gynaecology with the design of microconvex transducers, as explained earlier (Gelly, 1992).

Endorectal ultrasonography of the bovine and equine reproductive tract, requires transducers of several frequencies (3.5, 5 and 7.5 MHz), depending on the detail required and the stage of pregnancy to be studied. Experience in these species has shown that a 7.5 MHz transducer is preferred to scan the ovary and early conceptus, with the lower frequencies employed when greater penetration is required, 5 MHz for over 40 days pregnancy and 3.5 MHz in late pregnancy (Boyd and Omran, 1991). The situation is similar for transrectal scanning of the pregnant uterus in sheep. Authors have reported the use of 5 MHz and lower frequency transducers at later stages of pregnancy (Buckrell, 1988; Kähn, 1994). A 7.5 MHz transducer has been used transrectally for ovarian studies in small ruminants, (Dorn et al., 1989; Schrick et al., 1993; Ravindra et al., 1994) or

in the autotransplanted ovary, under the skin of the neck (Campbell et al., 1991a&b, 1994), but a superovulatory response is also detectable with the lower frequency transducers (Bor et al., 1992).

Transrectal scanning technique

There are no probes developed specifically for endorectal use in sheep, therefore various authors have adapted small probes designed for other species to this use. Some have introduced the probes within a rod extension to allow external manipulation (Kähn, 1994). Others report the use of human transducers (Schrack et al., 1993), which have a hand piece to allow positioning of the probe (Spence et al., 1994). The maximal dimensions of the probes that can be introduced into the rectum of sheep are: 10-15 cm length, 3 cm height and 2 cm width (Kähn, 1994). The animal should be properly restrained to avoid injury and the procedure is well tolerated without the need of sedation or anaesthesia. The probe is lubricated with Lubrol and then introduced for about 15 cm into the rectum, where the urinary bladder should become visible. Once the bladder has been identified, the probe is advanced and rotated ventrally and laterally to image the uterus. Faeces are removed from scanning image, by moving the probe back and forth or reintroducing it (reviewed by Kähn, 1994).

Interpretation: terminology and artefacts

On an ultrasound image, fluid does not reflect sound waves and appears black on the screen, this is called anechoic, non-echogenic or electrolucent. The content of the urinary bladder is normally completely anechoic and should be differentiated from other anechoic structures belonging to the reproductive tract: follicular fluid, embryonic vesicle and lacunas that may exist within the *corpus luteum*. On the other extreme, bone (pelvis and foetal bone) and the dioestrus cervix strongly reflect the sound waves and appear

white on the screen, being termed as hyperechogenic. The echo patterns produced by the different tissues are characteristic, based on the cellularity and stromal composition, (increased stromal composition results in increased echogenicity). Soft tissues (*corpus luteum* and endometrium) reflect and attenuate sound waves to different degrees producing images of varying shades of grey, being termed echogenic with different echotexture. Other organs in the pelvic cavity like the hyperechoic bowel gas or fluid filled structures and bones can be sources of artefacts (reviewed by Boyd and Omran, 1991; Ginther, 1986).

The most commonly found artefacts in the reproductive tract have been reviewed in large animals by Boyd and Omran (1991), Ginther (1986) and they are the following:

1) Specular reflection

An enhanced echo signal is received when the sound waves meet an extensive, smooth surface at the right angle. Examples: amniotic sac, surface of large follicle and luminal surface of the vagina.

2) Scatter reflection (non specular)

This occurs when the sound waves are reflected at different angles from small interfaces, producing a speckled appearance. Scatter causes the majority of diagnostic echoes in cellular organs. Examples: image from a transducer submerged in tap water, the scattering is due to small air bubbles and the differentiation of a mature *corpus luteum* from the rest of the ovary.

3) Reverberation

The sound waves are bounced back and forth between two reflective interfaces causing repetition of echoes.

Example: pneumovaginitis and gas-filled loop of bowel.

4) Acoustic enhancement

Structures deep to a fluid (anechoic) structure appear hyperchoic, which means more echogenic than its normal echotexture.

Example: follicles

5) Acoustic shadowing

Structures lying deep to hyperechoic structures (bone, etc.) appear as black.

Examples: pelvis and cervix can cause this artefact.

Ultrasound imaging of the cervix and other structures in the pelvic cavity

Cervix, urinary bladder and pelvic bone

Attempts to use ultrasound to study the anatomy of the ovine cervix at oestrus were not successful in imaging the cervical canal *in vivo* and not enough anatomical detail was obtained in the water bath (Halbert et al., 1990a). There is no description of the ultrasonographic appearance of the ovine cervix in the literature. For the schematic topographical anatomy of the reproductive tract of the ewe see Plate 5.3A.

In the bovine the cervix is anatomically similar to that of the sheep, in shape and localisation within the pelvic cavity (Hafez, 1973; Dyce, 1987; Ashdown and Done, 1984; Hafez, 1993), a cross section of the cervix is obtained by placing the transducer on the floor of the rectum and over the cervix, following the longitudinal axis of the animal. On ultrasound examination, the cervix of the cow appears as a distinctly echogenic structure with various degrees of acoustic shadowing due to the high echogenicity of the cervix and especially its mucosal folds or rings. The bovine cervix can be found beneath the plica circularum of the rectum and above the hyperechogenic pelvic bone. Another landmark is the urinary bladder which is found lying cranial in the animal and appears as an

hypoechoogenic (black) area beneath the cervix. In ruminants, a full bladder extends cranially along the abdominal floor due to distension and retreats within the pelvis when empty (Dyce et al., 1987). A full bladder therefore shifts to the left of the screen (cranial) in an ultrasonogram (Omran, 1989). The appearance of the wall of the bladder changes according to the degree of filling. The wall is less obvious with a full bladder, as the distended wall is thin and produces only a few echoes. The external os of the cervix is visible but the internal os is difficult to image as it appears as an indistinct structure from the uterus proper. The cervical canal can be seen lying horizontally in the centre of the cervix. The zigzag course of the canal which appears anechoic is visible by rotating the transducer through the longitudinal axis relative to the canal (Omran, 1989).

Ginther (1986) reports changes in echotexture of the cervix in the mare, depending on steroids levels. The echogenicity of the cervix is similar during dioestrus and pregnancy and this is due to tissue density. When the cervix becomes flaccid during oestrus, it loses its hyperechogenicity and is difficult to differentiate from surrounding tissues. Changes in echogenicity relating to cervical softening have also been noticed in women (Andersen and Ansbacher, 1991).

Vagina

There is no description of the ultrasonographic appearance of the ovine vagina in the literature. In the bovine, the collapsed walls of the cranial vagina are seen as an hyperechoic line in the longitudinal axis. When the vagina is fluid filled, its lumen appears ovoidal and anechoic with hyperechoic lines (Omran, 1989). Cervical mucus at oestrus is highly echogenic (Beal et al., 1989).

Non pregnant uterus

The non-pregnant uterus of the ewe can be found inside the pelvis at the region of the apex of the urinary bladder. Using transrectal sonography, a sagittal section is obtained if the transducer is held dorsally over the uterus. The cranial border (greater curvature) can be differentiated as a convex structure. Normally there is no fluid within the uterus, and the echogenicity is homogeneous (Kähn, 1994).

Ovaries

There is limited experience in the ultrasonographic imaging of the ovaries in the ewe. Images of normal ovaries have been published using excised specimens in the water bath and employing transrectal ultrasound (reviewed by Kähn, 1994). The ovary can be found cranioventrally to the urinary bladder and ventral to the non-pregnant uterus. The echogenicities of follicles and *corpora lutea* (CL) are similar to the ones observed in cattle (Boyd and Omram, 1991; Omram, 1989; Kähn, 1994). The *corpus luteum* is hypogenic compared to the rest of the ovary, as in cattle the CL can present fluid filled cavities (lacuna) that appear anechoic. Lacunas in the CL may be confused with small follicles, which appear round and anechoic, because the wall of the CL can be as thin as 1-2 mm. The size of the follicles and CL in these species can be sometimes too small (2 mm) for the resolution capacity of the transducers usually available for veterinary use. Transrectally, only when the follicles reach more than 4 mm, because of superovulation or pathological conditions (cystic ovaries) can the ovary be found and follicles identified with certainty. Preovulatory follicles, when they reach 5 mm or more, can be identify sometimes during normal ovulations.

5.1.4 Aims of the work

Ultrasonographic studies in large animals have shown the potential of this technique (Ginther, 1986; Boyd and Omram, 1991; Griffin and Ginther, 1992), in small ruminants similar studies are beginning to be possible using new transducers with better resolution (Kähn, 1994). It is possible that the ultrasonic appearance of the whole reproductive tract could also be monitored in sheep in the near future, using the appropriate probes and scanners. The imaging of the cervix may be helpful in the development of transcervical techniques of AI and ET.

The aims of this work were:

- 1) To test whether the ovine cervix could be imaged using the endorectal technique initially employing a probe of 5 MHz, originally designed for both endorectal or intravaginal use in humans (Plate 5.2A). Later, to obtain better resolution, a 7.5 MHz microconvex intraoperative probe was tested (Plate 5.2B).
- 2) To observe possible changes in the echogenicity, canal patency and dimensions of the ovine cervix during proestrus, oestrus and the early luteal phase.

5.2 MATERIALS AND METHODS

In the first experiment, the cycle was synchronised in 6 ewes during the breeding season, using progestagen sponges for 13 days (see Materials and Methods in Chapter III). Sheep were scanned standing without sedation or anaesthesia and were restrained during the scanning with two bales of straw and one person. Two sonographers scanned the sheep, one operated the scanner and the other the endorectal probe. Sheep were scanned at oestrus (n=4 ewes) and during the early luteal phase (n=2 ewes) using a endorectal 5 MHz linear array probe (Toshiba) and a linear scanner (Capasee, Toshiba) (Plates 5.1 and 5.2. A). The insertion tip of the probe was sheathed and distilled water was injected into the sheath. The probe was lubricated with Lubrel and gently inserted into the rectum (Figure 5.3 A). Further water was injected to allow good contact between the probe and the wall of the rectum.

The 5 MHz Toshiba Intracorporeal probe (model IVE-506S transducer) was originally designed by manufacturers for the examination of internal male and female reproductive organs in humans and has been used successfully for the diagnosis of prostatic disorders in the dog (Boyd, personal communication; Spence et al., 1994). The selection of frequency and the amount of distilled water used were based on previous experience in the dog studies. The probe can be focused to different depths, which is an advantage for the endorectal scanning of small species.

In the second experiment 12 anoestrous sheep were used. Oestrus was induced and synchronised in all of them with progestagen sponges and an intramuscular injection of 500 I U of eCG. Three groups of sheep (n=4, on each group), namely proestrous, oestrous and early luteal phase (day 7 after oestrus) were scanned transrectally on dorsal recumbency, restraining them in a rollover crush (Fig 5.3B). The ultrasound equipment utilised were a Toshiba Capasee scanner (Plate 5.1) and a 7.5 MHz microconvex intraoperative probe (microprobe PVF-738F, Toshiba) mounted in a plastic

introducer (Plate 5.2B). This type of probe is used in human medicine to perform abdominal surgery (Paterson, personal communication) and has been successfully used transrectally to image the ovary in sheep in previous experiments (Dickie et al., 1997).

The scans were recorded on video tapes (Fuji, SuperVHS). Representative photographic prints were made using a colour video printer (Sony) feed with black and white paper and a video monitor (Panasonic) linked to ATL Apogee sector scanner.

Plate 5.1

Ultrasound equipment used in this study

The scanner was linear (Capasee, Toshiba)



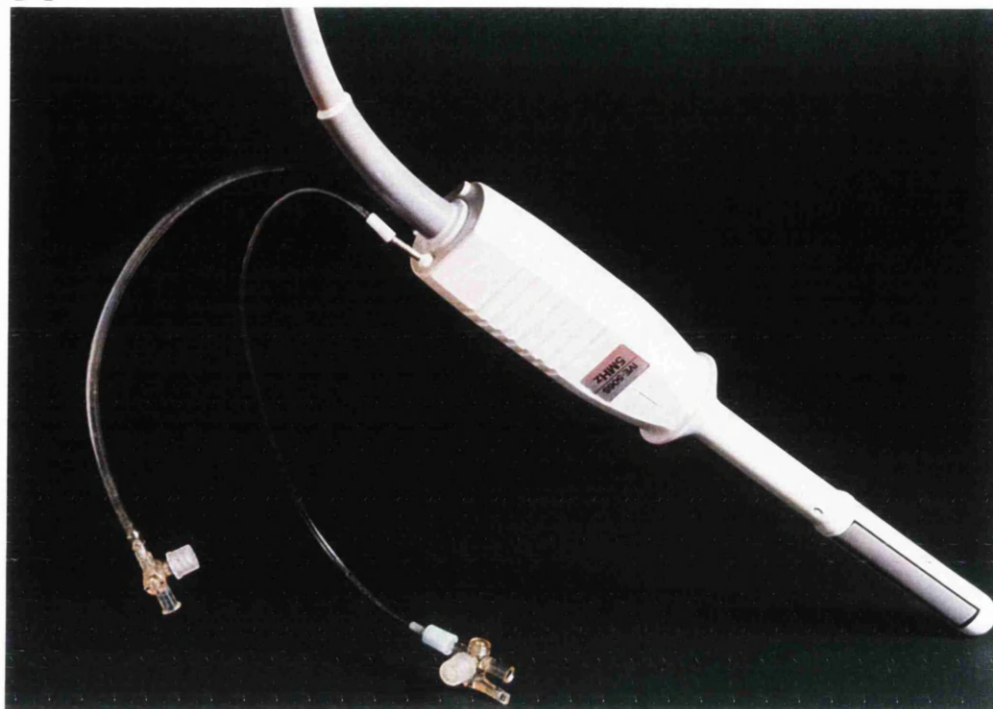
Plate 5.2

Ultrasound equipment used in this study

A. Endorectal linear array probe (intracorporeal IVE-506S, Toshiba) of 5 MHz

B. Intraoperative microconvex probe (microprobe PVF-738F, Toshiba) of 7.5 MHz

A



B

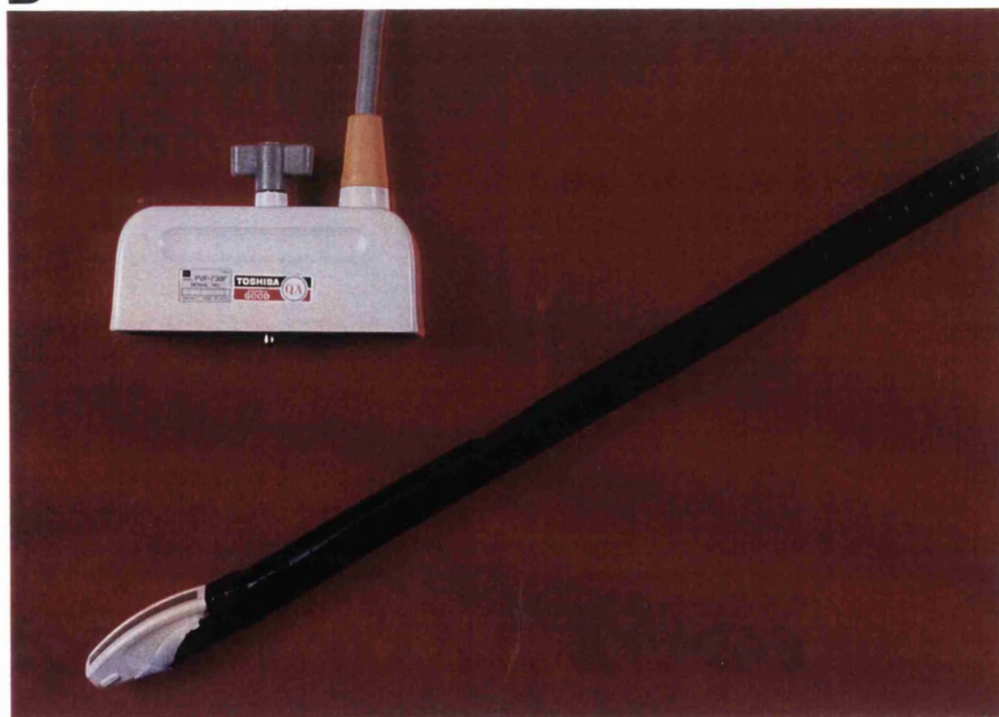


Plate 5.3

Ultrasound scanning techniques

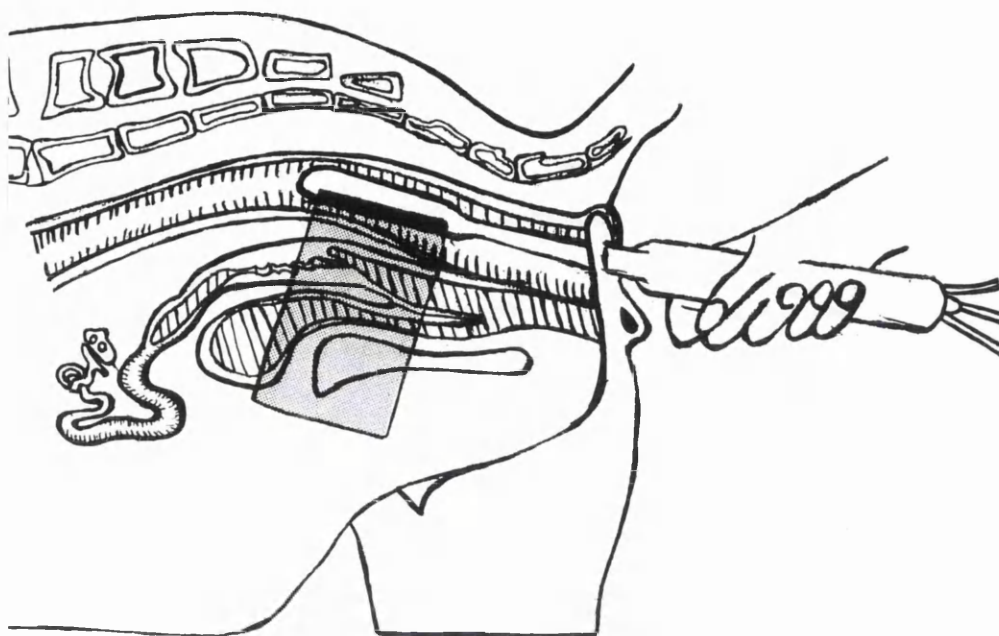
A. standing position

Schematic diagram showing topographical anatomy of the reproductive organs and the transrectal technique using the endorectal linear array probe (intracorporeal IVE-506S, Toshiba) of 5 MHz. Redrawn from Hafez (1993) and Kähn (1994)

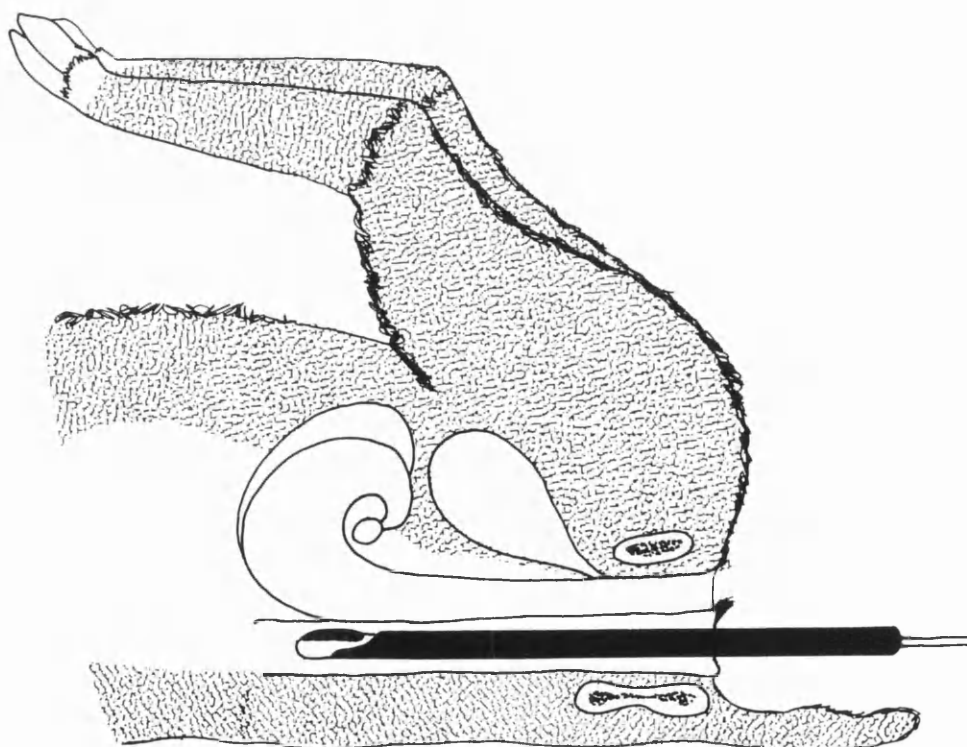
B. the recumbent position

This technique was used with the intraoperative probe (microprobe PVF-738F, Toshiba) of 7.5 MHz. Kindly donated by Alison Dickie and Calum Paterson.

A



B



5.3 RESULTS

It was possible to obtain ultrasonographic images of the cervix with the transrectal technique. Better image quality was achieved with the 7.5 MHz microconvex probe than with the 5 MHz linear probe and therefore only those images obtained with the former are shown (Plate 5.4). The external os and mid cervix could be clearly identified, but the internal os could not be readily differentiated from the uterine body. The external os of the cervix was identifiable due to the presence of fluid in the lumen of the cranial vagina, which contrasted with the hyperechogenicity of the cervix. The cervical canal was not clearly identifiable with ultrasound. The cervical length was difficult to measure with accuracy due to the tortuosity of the cervix which prevented accurate sagittal sections. A poorly defined junction of the internal os with the uterus, also prevented accurate measurements of the cervical length.

There was no generalised change in the echogenicity of the cervix throughout the cycle. There were changes in the amount of cervical convolution and the diameter. During oestrus the cervix appeared shorter and broader with less convolutions. During dioestrus the cervix appeared longer and more curved making it more difficult to image in longitudinal plane (Plates 5.4 A, B and C).

Plate 5.4

Sonograms of the ovine cervix

Images obtained with the 7.5 MHz microconvex rectal probe.

A. Proestrus

Left arrow points to the internal os and the right arrow to the external os. The external os was easier to recognise because of the presence of fluid in the cranial vagina. The cervix was recognisable because it appears hyperechogenic. Diameter is 2 cm.

B. Oestrus

Diameter is approximately 2.5 cm.

C. Early dioestrus

Cervix appears more echogenic and with a slightly narrower diameter (2 cm).

A



B



C



5.4 DISCUSSION

Rectal palpation is not possible in the ewe and the imaging of the tract may be extremely useful in all aspects of reproduction of this species. In this preliminary work, ultrasonograms illustrate the normal appearance of the ovine cervix during oestrus and the luteal phase. These images have not been published before. The images obtained in sheep were similar to those published of the cow's cervix (Beal et al., 1989; Omran, 1989; Boyd and Omran, 1991), except that the canal was not visible in the sheep. Changes in echotexture were not observable during the cycle in the sheep. Relaxation of the cervix, associated with changes in echotexture has been reported in mares (Day et al., 1995). It is not clear if a similar relaxation occurs in the ewe (Ward, 1968). It remains to be tested whether the canal and the differences in echotexture could be observed in sheep using a higher frequency probe.

If suitable catheters and techniques are developed to pass the cervix, ultrasound imaging of the ovine cervix may help to guide the catheter without damaging of the tract. In cattle, a method using transrectal ultrasound was developed to train artificial inseminators. A retrievable echogenic metal bead was deposited in the uterine lumen and its location determined (Beal et al., 1989). This method may be also applicable in the sheep.

Others have reported differences in size and echotexture related to stage of the cycle, stage of pregnancy and hormonal changes at preterm labour in other species (mares: reviewed by Ginther, 1986; Day et al., 1995; women: Andersen and Ansbacher, 1991; Vavra et al., 1993). A systematic characterisation of the echogenicity of the cervix at these stages, would allow the assessment of the cervix following the administration of ripening drugs. Ultrasound used to assess cervical status, has several advantages over other methods, it is non-invasive, recordable, repeatable in the same animal

and can be objective and quantitative. Ultrasonographic studies can be correlated with microscopical, mechanical and biochemical studies of the cervix and also with the presence of receptors and the peripheral levels of hormones. These applications may need to wait until the technology permits this type of studies in sheep.

Conclusions:

1) The ultrasonographic appearance of the cervix at oestrus has not shown whether there is relaxation of this organ, as in other species (Day et al., 1995). This has been controversial in sheep (Ward, 1968) and its clarification is important for the development of a transcervical AI technique.

2) The echotexture of the luteal phase cervix was not characteristic of this stage, when compared to anoestrus or oestrus sheep. Using higher frequency transducers, higher resolution may be achieved which allows comparison of echotectures following ripening treatments at this stage to develop transcervical ET methods.

3) Endorectal ultrasound imaging of the ovine cervix, otherwise inaccessible, is possible. At the moment, the technique may be helpful for the development of transcervical techniques for AI and ET, possible applications are: (1) to test catheters and transcervical techniques of AI and ET, by monitoring the progress of a catheter in the cervical canal, (2) to ascertain the site of semen deposition, like in previous studies in cattle (Beal et al., 1988).

4) The endorectal probes were found to be useful not only to image the cervix, but also the ovaries with considerable detail. During the writing of this thesis, others have also performed ovarian sonography by using tansrectal scanning (sheep, goat and pigs: Ginther, 1995) or the autotransplanted model (Souza et al., 1996) and have studied follicular dynamics. These studies in conjunction with anatomical studies of the cervix, may have a great

impact in the improvement of MOET (Multiple Ovulation and Embryo Transfer) programs. Transrectal ultrasound can contribute to sheep welfare, helping to develop transcervical AI and ET techniques and substituting for laparoscopy in ovarian studies. It is envisaged that ultrasound may become a very valuable clinical and research tool in all aspects of the reproduction of the ewe and other small farm animals.

CHAPTER 6

GENERAL DISCUSSION AND FUTURE PROSPECTS

Since the late 1960s and 1970s, it has been known that both PGE₂ and PGF_{2α} play important roles in the expulsion of uterine contents in mammalian and avian species of veterinary interest, participating in parturition and ovoposition (reviewed by Jonston et al., 1990; Bahr and Johson, 1991). It is now known that in both groups of vertebrates, local PGE₂ is superior to PGF_{2α} when used clinically to dilate the cervicovaginal region in cases of dystocia. Human obstetrics have exploited the effects of PGE₂ extensively, but this has not happened in veterinary obstetrics. Human preparations have been shown to be effective in several vertebrate species, but there is no commercial preparation of PGE₂ for veterinary use (Arthur et al, 1996). The only drugs indicated for the induction of labour are corticosteroids, for a faster delivery of the foetus or the egg, usually oxytocin and some times PGF_{2α} are used (Jackson, 1995). The use of these oxytocics with an undilated cervix however, may lead to uterine rupture or shell gland rupture (mammalian: reviewed by Dobson, 1988; avian: reviewed by Hudelson and Hudelson, 1996).

Many clinical applications can be found in theriogenology for PGE₂ across the whole spectrum of companion, livestock and wildlife animals. Interesting indications for PGE₂ in female reproduction may be: 1) the induction of labour and pregnancy termination in mammals, 2) the induction of ovoposition in birds and reptiles, 3) treatment of ringwomb, mainly in ruminants, 4) treatment of egg-bound birds and reptiles and 5) obstetrical interventions in non-gestating females that require access to the uterus, as for example transcervical AI and ET in ruminants.

In human obstetrics, the use of PGE₂ is more sensible than performing a caesarean section, for economical reasons (Aughey, personal communication). This has not been investigated in veterinary obstetrics, but caesarean sections are not cheap in domestic animals either. In the case of

the sheep, as an example of the lower range in price, this operation cost an average of £50. The cost of prostaglandins of the E series ranges from £4 to £26. Apart from the cost, the caesarean section requires aseptic conditions, sedation and anaesthesia, this may cause unnecessary delay for some emergencies. In very large species, like elephants, a caesarean section is a major and highly risky operation that requires general anaesthesia and can take several hours. In addition, there are very few vets in the world with experience in these animals, which adds to the cost and does not guarantee that the cow is going to survive the operation (Lyons, 1997). Eggbound problems in birds and reptiles can be also life threatening, since eggs broken within the abdomen can cause a fatal peritonitis (Hudelson and Hudelson, 1993; Jackson, 1995). The development therefore of suitable drugs that facilitate birth in animals, will mean a very important improvement in welfare for several species.

The experimental work in this thesis was focused on the use of intravaginal human preparations of PGE₂ to facilitate the development of transcervical methods for ET in sheep. With this objective in mind, several aspects of cervical ripening have been covered in the literature review and investigated (chapters I, III, IV and V). In chapter I, the use of hormones to facilitate cervical ripening at several reproductive stages is reviewed to illustrate that PGE₂ is not the only drug that can be used to induce cervical ripening. Several aspects of physiological ripening at term have been reviewed because presumably these type of changes may be inducible with PGE₂ or other hormones during the cycle. PGE₂ appears to be the most important prostaglandin for the dilatation of the cervix and vagina, participating not only in the mammalian birth and avian ovoposition, but also in fish spawning and egg laying of insects (Loher, 1981; Staceyne, 1976; Urich, 1990; Evans, 1993), which suggests that this function may be conserved between vertebrates and some invertebrates. This is important to

support extrapolations of PGE₂ within mammalian species, often used in this thesis. However subtle differences may be expected, specially within the lower taxa.

The structure of PGE₂ and its function in the cervix are well conserved certainly within mammalian species and also within many vertebrate species (Toh et al., 1995; Bowman et al., 1996). This is interesting for the veterinary profession, which, as indicated earlier has to deal with a wide range of species. In contrast, relaxin is unusually poorly conserved between different mammalian species, therefore it has been difficult to study its function in species like sheep and cattle (Sherwood, 1994). Since, porcine relaxin appears to induce cervical ripening in these species (see chapter I), it is possible however that the receptor is more conserved than the ligand, which may offer an opportunity to study the role of relaxin in new species. Although oxytocin is used in obstetric cases of ancient species like reptiles, oxytocin seem to have appeared between the transition of marsupials and true mammals, since some marsupials produce oxytocin, others produce mesotocin or both and birds produce arginine vasotocin, rather than oxytocin (Hueldenson and Hueldelson, 1993; Ivell and Russell, 1996; Renfree et al., 1996). The conservation of PGE₂ functions within bird and mammals in the cervix therefore appears to be quite unique and offers the advantage of being effective in a very wide spectrum of species of veterinary interest.

The objective of chapter IV was to gain more insight in the understanding of the mechanisms of action of PGE₂ in the ewe's uterine cervix and its possible applications for transcervical ET. A cDNA fragment encoding for the ovine prostaglandin receptor subtype EP₃R was isolated by RT-PCR. The nucleotide sequence of this fragment was highly homologous to the bovine (98%) and other mammalian species (80%). This is an expected result because the prostanoid receptor genes, like most GPCRs

are well conserved between mammals (Ismaa et al., 1995; Toh et al., 1995). mRNA expression studies appear to confirm the identity of the receptor and also that it may have a function in the cervix. Obviously the full length of the cDNA, may have slightly higher or lower homology with other mammalian sequences, than the fragment isolated here.

The effect of PGE₂ in the cervix will be better understood with the study of the EP receptors cDNAs and genes. The present studies on the ovine EP₃R are only the beginning of the characterisation of PGE₂ receptors in the ovine cervix. The cloning and expression of all the ovine EP receptors (EP₁₋₄) cDNAs and genes will lead to the availability of better reagents: oligoprimers, recombinant cDNA, gene and protein and antibodies, which would allow the elucidation of the physiological role of PGE₂ in cervical ripening. This could be beneficial for both human and veterinary obstetrics, leading to more specific treatments. Hopefully if the pharmaceutical and agricultural industries find a common interest, suitable preparations of PGE₂ analogues for ET in small ruminants, and the other purposes discussed above, may be developed. The availability of those molecular biology reagents, may also pave the way for physiological studies in other vertebrate species.

Interestingly, studies on the molecular evolution of the eicosanoid receptors, by computer-assisted sequence comparison of their receptors, suggest that the products of the cyclooxygenase pathway (PGE₂, etc.) and their corresponding receptors may have been established before mammalian divergence (Toh et al., 1995). It would be interesting therefore to assess the role of PGE₂ and its four receptors (EP₁₋₄) in the ovoposition of avian and other more primitive species, like fish and reptiles for both evolutionary and clinical purposes, since some of these species are gaining importance in the veterinary profession. Prostanoid receptors have only been cloned in mammalian species, but there is evidence for the existence of EP receptors

in avian species and amphibians (Lefkowitz et al., 1977; reviewed by Hudelson and Hudelson, 1996). Although we do not know how old the prostanoid receptors are, some parasitologists think that even invertebrates (nematodes, ticks, etc.) and lower organisms like bacteria or yeasts (Belley and Chadee, 1995; Bowman et al., 1996; Tizard, 1996; Stanley Samuelson and Pedibhotla, 1996), can produce prostaglandins, indicating that they are well established early in the evolutionary tree.

It may be possible to use mammalian sequences of the prostaglandin receptors to amplify by PCR the homologous receptors in other vertebrates. Within vertebrates GPCR homology can range from 60% to 90% and cross-species cloning has been done between species sharing the lower homology (Hall et al., 1993; melatonin: Reppert et al, 1994). Studies of the 3'end of the EP₃R in avian and reptiles species, may reveal when in evolution the alternative splicing occurred, originating the receptor isoforms that have been reported in all mammalian species. Possible modifications of the techniques used here (RT-PCR and RACE), have been pointed out for cloning of EP₃R and its isoforms in other livestock, companion and exotic species. As far as the veterinary profession is concern, the role of PGE₂ in ovoposition may also be worth tracking back, to treat eggbound problems nonsurgically in exotic pets or for conservation purposes. Blocking prostaglandin receptors may be a vaccine strategy against those parasites that use the secretion of prostaglandins to avoid host defences (Bowman et al., 1996; Tizard, 1996). Different veterinary interests may be joined in the characterisation of the EP receptors or its evolutionary forerunner of several vertebrates and invertebrates; not only in the in the cervix but also in the immune system which together may justify economically these investigations.

In chapter IV, mRNA studies also show that the ovine EP₃R, like in other species, is also implicated in the function of other organs that are not relevant for the topic of this thesis (ovary, kidney, uterus, adrenal gland, skin and liver, etc.), but may have an interest in other disciplines, like reproductive biology, internal medicine, parasitology and immunology. Here the hypothesis that EP₃R is a proinflammatory receptor is supported, which if confirmed could be used therapeutically. Some parasitologists think that PGE₂ produced by parasites is immunosuppressive in the host and in this case antiparasite drugs may be developed by blocking receptors in the host.

It is remarkable that there are very few studies of PGE₂ receptors in the cervix, despite the recognised importance of PGE₂ in its function. This situation has not changed during these studies, despite a gradual increase in the availability of cDNA and gene sequences for these receptors in several species. In contrast, these reagents have been rapidly applied to the clarification of other ovarian and uterine actions, in the endometrium and myometrium. The lack of similar studies in the cervix is probably due to the incompleteness of early studies on the characterisation of EP receptors in cervical tissue through the cycle and pregnancy. Studies on other tissues (ovary, endometrium and myometrium), are taking advantage of more detailed binding sites studies, which previously had shown increases in the density of these receptors at certain stages and in some cells types. Second messenger studies in those tissues are also more informative, not being restricted to the study of cAMP and giving clues of which receptor subtypes may be involved. In the cervix, therefore these basic questions need further clarification.

A recent publication has concluded that cAMP is involved in changes in the secretion of GAGs by fibroblasts *in vitro*, during cervical ripening (Carbonne et al., 1996). This and other early works may indicate the implication of cAMP and therefore the EP₂ or EP₄ receptors in cervical

ripening (Norström et al., 1983). However, PGE₂ is expected to have different signal transduction pathways and effects in different cells, and in the cervix many cell types, apart from the fibroblast, are potential targets. Furthermore, cAMP had been known since the late 1950s and the phospholipase C, has only been discovered in the early 1980s (Ismaa, 1995), therefore cAMP was regarded as the only second messenger for PGE₂ in early studies. During the 1990s, some works have confirmed a role for a transduction pathway positively coupled to cAMP in the smooth muscle of the cervix in mammals, but in the avian cervix, low physiological concentrations are negatively coupled with cAMP (Molnár et al., 1987; Norström and Bryman, 1991). Others, using fibroblasts in culture, have found evidence against the role of a receptor positively coupled with cAMP, and have shown an involvement of the phospholipase C pathway (Takahashi et al., 1991 and 1993; Ito et al., 1991 and 1992; Rajabi et al., 1992). Some of these works, in mammalian fibroblasts and avian smooth muscle, would suggest an implication of the EP₃R in cervical ripening. In the cervix, there are no *in vitro* studies on the transduction pathways of the immune cells, which may be hypothetical alternative targets for PGE₂ (Uldbjerg, 1989; Knudsen et al., 1991; Rath et al., 1993).

In chapter III, activation of several immune cells (eosinophils, PMLs, platelets and mast cells) was observed following the administration of PGE₂. These cells were clearly responding to the administration of PGE₂, this is consistent with the inflammatory theory of cervical ripening and the proinflammatory action of PGE₂. Research into the role of EP receptors in the functions of immune cells is very active at the moment, and in this type of *in vitro* study the EP₃R receptor has been implicated in the activation of platelets, PMLs and mast cells (Wheeldon and Vardey, 1993; Nishigaki et al., 1993; Matthews and Jones, 1993; Blockmans et al., 1995). Because these cells participate in the mechanisms of physiological ripening at term, it is

suggested here that further studies on this receptor in the cervix may be productive. According to the hypothesis sustained in this thesis, EP₃ analogues would be suitable for the induction of cervical ripening. A complete EP₃R agonist is not available yet, but it may be interesting to test the classic EP₃R agonists in the ovine cervix (Sulprostone, Misoprostol and Gemeprost) and other more specific that are being developed by the pharmaceutical industry. Obviously transgenic 'knock-out' mice of PGE₂ and its receptors or the discovery of specific PGE₂-associated pathologies will be very informative. Given the importance of these receptors in many physiological roles, apart from reproduction, it is possible that these transgenic animals may be available in the near future. Several groups are carrying out structural studies of EP receptors, using site directed mutagenesis, this may lead to the elucidation of the three-dimensional structure of these receptors which will ultimately could allow for the design of more specific agonists and antagonists.

Immunological research about the role of EP receptors in the functioning of cells is also looking into the stimulation of the synthesis of matrix metalloproteinases in several cells, although it is still not clear which receptor subtype(s) are implicated on each cell; several subtypes have been implicated in the synthesis of MMPs, including the EP₃R (Zeng et al., 1996). Theories about the involvement of the EP₂R in vasodilatation have been mainly investigated in the skin (Armstrong et al., 1995). According to these hypotheses, this receptor could also be implicated in cervical ripening (Kelly, 1996). Implantation has also been compared with an inflammatory response, and both the EP₂R and the EP₃R, appear to be upregulated in the endometrium, but it is too early to know the precise role of these receptors (Katsuyama et al., 1997; Yang et al., 1997). In conclusion, other studies are compatible with the initial hypothesis of the involvement of the EP₃R in cervical ripening. But the role of the EP₃ and EP₂/EP₄Rs, in the different cell

populations of the cervix may be further clarified using immunocytochemistry and *in situ* hybridization.

Chapter V deals with the ultrasonographic imaging of the ovine cervix. During these studies there have been considerable advances in the ultrasonographic study of the reproductive tract in the sheep. It would appear that, as in cattle, equipment for the examination of small ruminants will become commonplace in institutions dedicated to ET in sheep, to monitor the ovarian response. This technique may also be very useful to guide intracervical catheters and to monitor cervical responses to ripening drugs.

Thus, the combination of ultrasound studies with recombinant DNA technology could advance our understanding of the pharmacological induction of cervical ripening in sheep to develop transcervical techniques. The identification of the main receptors involved in the physiological inflammatory response that occurs during cervical ripening, could lead to a more specific pharmacological intervention in the reproductive and in the immune systems.

Molecular studies of prostaglandin receptors in non mammals is the next challenge, this is interesting for the scientific community in general, to know about the evolution of EP receptors. In addition, this could lead to reproductive physiology studies and to pharmacological intervention during late pregnancy to facilitate labour or ovoposition in a wide range of species of veterinary interest.

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AUDICANA L., AUGHEY E. and O'SHAUGHNESSY P. J.

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